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PRELIMINARY STUDIES ON BLOOD ACTH IN MAN*

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ASTWOOD's oxycellulose procedure (1) for the isolation of pituitary ACTH as modified by Bartholomew[1] (2) has been applied to the analysis of blood ACTH in man. The details of the technic have been described elsewhere (3).

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[1] Due to an unfortunate oversight, Dr. Bartholomew was not given credit in the paper by Sydnor and Sayers (3) for modifying the oxycellulose technic of Astwood. In the technic as described by Payne *et al.* (17) ACTH is precipitated from acetic acid solution by the addition of acetone and ether. Bartholomew (unpublished results) devised a technic which avoids precipitation with organic solvents. The glacial acetic acid extract of pituitary is diluted with water to make a concentration of acetic acid less than 1.0 N. Super-Cel is added to clarify the extract, followed by oxycellulose which adsorbs the ACTH. Sydnor and Sayers (3) applied this modification to the analysis of blood ACTH. Recently it has been found that it is not necessary to use Super-Cel in processing blood.

METHODS

Blood was obtained from 15 subjects: 7 healthy adult males, 2 patients with active pulmonary tuberculosis, 3 patients with Addison's disease, 2 patients with Cushing's syndrome (a female with bilateral adrenal hyperplasia and a male with carcinoma of the adrenal gland), and 1 patient with anorexia nervosa.

A blood sample was obtained from a vein in the antecubital fossa and immediately mixed with 4 volumes of glacial acetic acid. The blood-glacial acetic acid mixture was processed by the oxycellulose technic for the determination of ACTH (3) and assayed in male hypophysectomized rats by the adrenal ascorbic acid-depletion method (4). Freshly prepared ACTH standard solutions, 0.25 and 1.0 milliunit[2] or 0.25, 0.5 and 1.0 milliunit per 100-gram assay rat, were tested with blood eluates in every assay. The number of rats injected with a given sample was determined by the total volume of blood available and by the dose per rat. For example, if the volume of the blood sample was 120 ml., the eluate was divided into three equal parts and assayed at a dose equivalent to 40 ml. of blood per rat.

RESULTS

The results are expressed in terms of adrenal ascorbic acid-depletion, that is, the concentration of ascorbic acid in the left adrenal, removed immediately prior to injection, minus the concentration of ascorbic acid in the right adrenal, removed one hour after injection of the test material. A negative value indicates that the concentration of ascorbic acid in the right adrenal is higher than that in the left adrenal. Fluctuations about zero for the difference between the concentration of ascorbic acid in the left and in the right adrenal following the injection of 0.9 per cent sodium chloride solution represent, in part at least, the error of the analytical technics employed. Biological variation may also contribute to this fluctuation about zero. Results of an experiment in which 0.9 per cent sodium chloride solution was injected are as follows: −40, 10, 7, −3, 8, 17, 12, 47, 12, 7, −13, −19, −12, 17, −18, 24, −11, −18, −15, −6, 24, 12, 2, 9, 30, −17, 9, 18, −10, average *3*. The individual and average responses of the test rats to given doses of blood, together with the corresponding individual and average responses to the low dose of standard tested on the same day, are presented in Table 1.

A dose of 25 to 50 ml. of blood obtained from control subjects, febrile patients with active pulmonary tuberculosis, patients with Cushing's syndrome, and a patient with anorexia nervosa, failed to induce a signifi-

[2] One milliunit equals one thousandth of a U.S.P. unit of ACTH, which is defined as the activity of 1 milligram of the International Standard ACTH (La-1-A).

TABLE 1. BLOOD ACTH IN MAN

	Subject; sex; age (yrs.)	Dose of blood per rat (ml.)	Adrenal ascorbic acid depletion (mg./100 Gm. fresh adrenal)		Adrenal ascorbic acid depletion in response to 0.25 milliunit ACTH std. (mg./100 Gm. fresh adrenal)	
			Individual responses	Avg.	Individual responses	Avg.
Normal, non-stressed	H.B., male, 42	40	−28, −14	−21		
	L.S., male, 27	50	21, 36, 35	31	108, 104, 87, 60, 95, 91, 58	89
	J.T., male, 40	45	−8, 20, −15, −5	− 2		
	P.S., male, 25	45	−8, −43, −18, 25	−11		
	A.G., male, 42	45	48, 36	42	111, 73, 51, 52, 78	73
	G.S., male, 38	50	−16, −1, −25, 38	− 1	63, 36, 47, 32, 31, 35	41
	J.H., male, 18	25	21, 47	34	56, 91, 31, 16	49
Addison's disease prior to therapy	D.R.,* male, 51	25	64, 49, 68	60	25, 67, 42, 84	55
	H.B., male, 28	25	58, 112, 92, 63	81		
	Y.O.,* male, 43	25	173, 262, 211, 212	215	37, 10, 21, 68, 90	45
Addison's disease during therapy	D.R.,* male, 51 (DCA)	25	75, 78, 67	73	111, 73, 51, 52, 78	73
	H.B., male, 28 (cortisone)	25	95, 102, 96, 95	97	51, 93, 40, 16	50
	H.B., male, 28 (cortisone)	25	93, 160, 114, 158	131	17, 39, 65, 73	39
Pulmonary tuberculosis, febrile, without Addison's disease	C.H., male, 68	25	−5, −10, 18, −8	− 4	6, 40, 79, 51, 27, 22	38
	R.K., male, 48	25	16, −35, −14	−18		
Cushing's syndrome	R.V., male, 43	22	−20, −39, −65	−41	25, 67, 42, 84	55
		25	−6, −2, 25	6	111, 73, 51, 52, 78	73
	S.L., female, 40	25	51, 4, 12, 46, −5	22	25, 29	24
Anorexia nervosa	A.P., female, 48	40	11, −1, 17, −35	− 2	8, 12, 74, 11, 28, 63, 77, 22	37

* Patients with miliary tuberculosis.

cant depletion of adrenal ascorbic acid in the test animals. With the technic employed, it may be estimated that the blood of these subjects contains less than 0.5 milliunit of ACTH per 100 ml.

Blood from each of 3 patients with Addison's disease did bring about a significant degree of adrenal ascorbic acid depletion when assayed at the 25-ml. dose level. An equal degree of depletion occurred following the institution of specific therapy. Although the data indicate that the blood of patients with Addison's disease contains a higher than normal concentration of ACTH, the single-dose assay technic employed does not permit a definitive statement regarding the exact concentration.

DISCUSSION

Reported values for blood ACTH in normal nonstressed man vary from nondetectable quantities to 200 milliunits per 100 ml. of plasma. Results based on assays in intact animals may be dismissed without serious consideration, since the adenohypophysis may be stimulated to an increased

rate of release of ACTH by relatively mild changes in the external or internal environment of the test animal.

Bornstein *et al.* (5) reported that a total dose of 140 ml. of plasma from normal male subjects injected over fourteen days did not influence adrenal weight loss in hypophysectomized female rats. However, 250 ml. of plasma from hypertensive subjects did produce a small but apparently significant increase in adrenal weight over uninjected control animals. The plasma in these studies was concentrated by a modification of the Lyons acid-acetone technic (6).

A number of reports have appeared concerning blood ACTH in man, in which the adrenal ascorbic acid-depletion technic in hypophysectomized rats has been employed. Montanari *et al.* (7) claim that 0.5 ml. of plasma per 100-gram rat induces a significant depletion of adrenal ascorbic acid. They estimate normal plasma to contain 160 milliunits of ACTH per 100 ml. This same group (8) also claims that ultrafiltrates of plasma, injected in doses equivalent to 0.5 ml., induce a significant depletion of adrenal ascorbic acid. Cooke *et al.* (9), Bornstein and Trewhella (10), and Parrott (11) have used a modification of the Lyons technic to concentrate blood ACTH. The first group reported that 5 to 10 ml. of plasma from normal individuals induced a depletion of 50 to 100 mg. of ascorbic acid per 100 grams of adrenal tissue. The second group estimated that normal plasma has a concentration of 197 ± 39 milliunits of ACTH per 100 ml. and Parrott found a range of 59 to 103 milliunits of ACTH per 100 ml. On the other hand, Taylor *et al.* (12) did not find a detectable quantity of ACTH in 3 ml. of unconcentrated serum from normal subjects or from patients with Cushing's syndrome. Granirer (13) has stated that 2-ml. samples of fresh plasma from normal female subjects do not induce a significant depletion of ascorbic acid. The present study, in which the oxycellulose technic was employed, indicates that blood from normal nonstressed man contains nondetectable quantities of ACTH, *i.e.*, each 100 ml. contains less than 0.5 milliunit.

The question arises as to why Cooke *et al.* (9), Montanari *et al.* (7, 8), Bornstein and Trewhella (10) and Parrott (11) found ACTH in relatively high concentrations in blood from normal subjects, whereas other investigators have failed to detect it. The following explanations may be considered: 1) loss of ACTH activity during processing, 2) activation of blood ACTH, 3) instability of ACTH in blood or plasma, 4) nonspecificity of the assay method, 5) contamination of laboratory glassware with ACTH, and 6) assay technic.

1. *Loss of ACTH activity during processing.* The oxycellulose technic has been demonstrated to recover almost quantitatively both exogenous and endogeneous ACTH (3). On the other hand, no quantitative data have

been presented on percentage recovery of ACTH when blood is processed by the acid-acetone technic of Lyons. In this laboratory, the results of the acid-acetone technic have not been reproducible and it has not yielded quantitative recovery of blood ACTH.

2. *Activation of blood ACTH.* Blood ACTH may be present in an inactive state in the circulation. Treatment of blood with acid-acetone could conceivably convert the hormone to an active form. However, treatment of blood or plasma from normal human subjects with acid-acetone as described by Bornstein and Trewhella has invariably failed to yield active concentrates (14).

3. *Instability of ACTH in blood or plasma.* Unfortunately, no definitive data have been presented on the stability of endogenous blood ACTH. Work is in progress on this important phase of the problem. Preliminary data on the oxycellulose technic indicate that no appreciable loss of activity occurs before the sample is mixed with glacial acetic acid.

4. *Nonspecificity of the assay method.* It is impossible to provide incontrovertible proof that the adrenal ascorbic acid-depletion technic is specific for ACTH. In the authors' experience, various substances, toxic and nontoxic, have failed to induce depletion in completely hypophysectomized rats. Furthermore, injections of large volumes of blood or of blood concentrates from nonstressed normal subjects or from nonstressed experimental animals (intact or hypophysectomized) have invariably failed to induce adrenal ascorbic acid depletion. It is reasonable to assume that, of the various substances in blood, only ACTH depletes adrenal ascorbic acid. Additional experience with the oxycellulose method will test the validity of this assumption.

5. *Contamination of laboratory glassware with ACTH.* The sensitivity of the adrenal ascorbic acid assay method is about 0.25 milliunit of ACTH, which is equivalent to 0.001 microgram of Astwood's purified ACTH! It is obvious that extreme precautions must be taken to prevent contamination of blood or blood concentrates in laboratories in which potent preparations of ACTH are employed.

6. *Assay technic.* Good technic requires the assay of both unknown and standard at various dose levels on the same day in completely hypophysectomized rats. It is impossible to fulfill all these requirements in analyses of blood ACTH in man. The small amount of hormone present necessitates the use of a single dose of unknown. However, single doses of blood are assayed with multiple doses of standard on the same day. As an additional check on the validity of the method, it is planned to include a group of hypophysectomized rats injected with 0.9 per cent sodium chloride solution in each assay.

The preliminary data of this report are presented at this time because

of the extreme diversity of estimates of blood ACTH which have appeared in the literature. In agreement with the present findings in normal nonstressed man is the fact that the equivalent of 40 ml. of blood from normal nonstressed rats does not induce adrenal ascorbic acid depletion (15).

In Cushing's syndrome with associated bilateral adrenal hyperplasia one might expect to find an increased titer of ACTH in the blood. However, the patient (S. L.) with adrenal hyperplasia had no detectable ACTH in her blood when the latter was assayed at the 25-ml. dose level. No ACTH was detectable in the blood of R. V., a patient with adrenocortical carcinoma, a result to be expected if cortical steroids suppress pituitary ACTH activity. These data on patients with Cushing's syndrome are in agreement with those of Taylor *et al.* (12) but not with those of Bornstein and Trewhella (10) or of Parrott (11).

The blood of patients with Addison's disease contains detectable quantities of ACTH. These observations are in agreement with the findings of Taylor *et al.* (12) in man and with those of Gemzell *et al.* (16) and of Sydnor and Sayers (3) in adrenalectomized rats. The high titer of ACTH in hypocorticism is compatible with the concept that the cortical steroids inhibit ACTH release. However, in the present study, cortical steroid therapy did not appear to reduce the titer of ACTH in the blood of patients with Addison's disease, a result which does not support this concept.

SUMMARY

The oxycellulose technic of Astwood has been applied to the analysis of blood ACTH in man. Large doses of blood from normal nonstressed male subjects do not induce a significant depletion of adrenal ascorbic acid. Blood from normal nonstressed male subjects is estimated to contain less than 0.5 milliunit of ACTH per 100 ml. Blood from untreated and from cortical hormone-treated patients with Addison's disease contains detectable quantities of ACTH.

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BLOOD LEVELS OF 17-HYDROXYCORTICOSTEROIDS IN NORMAL PREGNANCY

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IT IS well known that the adrenals enlarge during the course of pregnancy. The enlargement is based on proliferation of the cortical cells. As it is reversible and disappears with the termination of pregnancy, it seems to be a purely functional alteration. There is little specific evidence to clarify its significance.

Methods have been developed whereby adrenal activity can be investigated indirectly by the measurement of substances in blood or urine derived from the adrenal cortex. Using a bioassay based on the ability of adrenocortical substances to cause deposition of glycogen in the liver of adrenalectomized mice, Venning (1) has studied the output of these substances in the urine of pregnant women and reported a marked increase, especially in the last trimester. Using chemical methods for the determination of the same urinary steroids, other workers (2, 3) have shown a similar rise to about twice the normal value during the third trimester. In 7 pregnant women in the eighth month of gestation, Cope *et al.* (4) have reported that the cortisone output in the urine was elevated.

It should be remembered, however, that at present there is an increasing awareness of the defects and limitations of the methods used for determining the excretion of adrenocortical steroids or their metabolites in the urine (5). It is also difficult to correlate these urinary substances with their precursors in the adrenal gland or to associate them with a particular type of adrenal function.

By using a method developed by Nelson and Samuels (6) it has been possible to determine the 17-hydroxycorticosteroids in the peripheral blood. To this type of adrenal hormone elaborated by the cortex belong compounds such as corticosterone, dehydrocorticosterone and their two 17-hydroxy derivatives. They all affect the carbohydrate and protein metabolism and at present there is much evidence available to suggest that these 17-hydroxycorticosteroids are the chief compounds secreted by the adrenal cortex.

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This paper is concerned with the blood levels of 17-hydroxycorticosteroids in pregnant women, in women just delivered and in women six days after delivery.

METHODS AND MATERIALS

Blood samples were drawn from 124 pregnant women. In all cases the duration of pregnancy was calculated from the first day of the last definite menstrual period. Blood was also drawn from 37 women immediately after delivery and from 10 women six days after delivery.

All these women visited the outpatient clinic of the department of Obstetrics and Gynecology at the Karolinska Hospital and were selected at random. However, patients exhibiting complications (albuminuria, high blood pressure and edema) were excluded. Those with an apparently normal labor were chosen for the blood samples taken immediately after delivery.

The concentrations of 17-hydroxycorticosteroids in the blood were determined according to a method described by Nelson and Samuels (6). Approximately 15 ml. of plasma was generally used and extracted with chloroform. The chromatography was carried out on magnesium silicate-Celite columns. The sample was dissolved in 0.2 ml. of methanol, 0.3 ml. of phenylhydrazine-sulphuric acid solution was added, and then incubated for one hour. After the incubation the sample was read in a 0.5-ml. microcuvette on the Beckman spectrophotometer at 370, 410 and 450 mμ. Correction for background absorption was made by using the absorption factor described by Nelson and Samuels (6).

RESULTS

The level of 17-hydroxycorticosteroids in the blood of young healthy nonpregnant women: The concentration of total 17-hydroxycorticosteroids in the plasma of 21 women in the same age bracket as the pregnant women of this study, was 6.6 ± 0.95 μgm. per 100 ml. Six days later, in the plasma of the same women the concentration of 17-hydroxycorticosteroids was found to be 5.2 ± 0.85 μgm. per 100 ml. In each subject, both blood samples were drawn at 9.30 p.m., *i.e.*, at about the same time as the blood samples were drawn from the pregnant women.

The level of 17-hydroxycorticosteroids in the blood of pregnant women: Figure 1 shows the concentration of 17-hydroxycorticosteroids per 100 ml. of plasma in the different stages of pregnancy. The dots represent the individual samples, and the line shows the increase which occurs during pregnancy. It was found, by using the method of least squares, that there is a positive correlation between the level of 17-hydroxycorticosteroids in the blood and the length of pregnancy (in weeks).

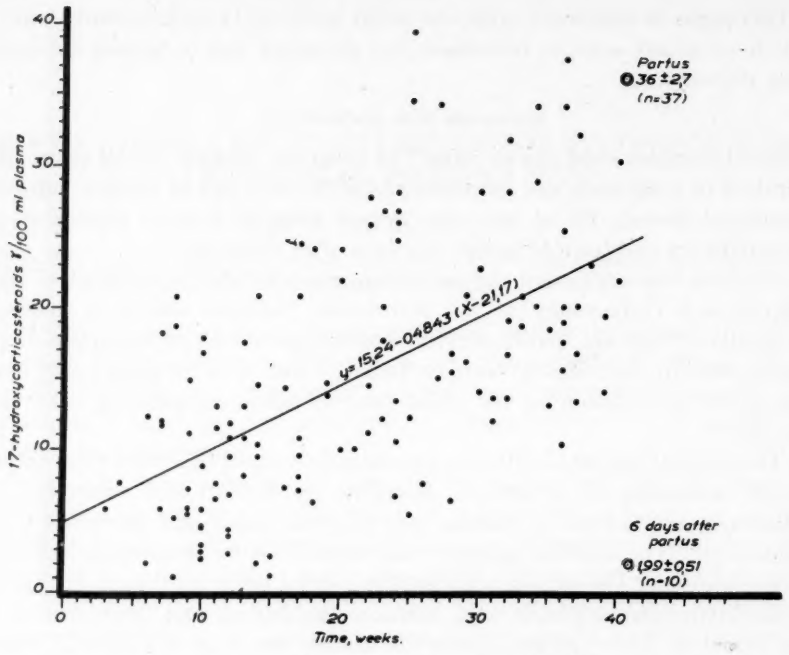

FIG. 1. Correlation between the concentration of 17-hydroxycorticosteroids in the blood of pregnant women and the duration of pregnancy (in weeks). Conception at zero time.

Analysis of variance for the data of Figure 1.

Source of variation	Degrees of freedom	Sum of squares	Mean square
Regression	1	308,640	308,640
Linearity	35	154,070	4,402
Residual	87	481,602	5,536
Total	123	944,312	

Test of regression $v^2 = 55.8$; *d.f.* 1.87
Test of linearity $v^2 = 0.8$; *d.f.* 35.87

It would appear from the analysis of variance that the regression is highly significant.

$$y = 15.24 + 0.4843(x - 21.17)$$

Coefficient of correlation:

$$r = 0.572$$

The regression line intersects the y-axis at a value of 5.0 μgm. per 100 ml. of plasma. This value may be considered as a normal one for young healthy women. During the last trimester of pregnancy the level of 17-hydroxycorticosteroids in the blood increases at least four times above the normal value.

The level of 17-hydroxycorticosteroids in the blood of women immediately after delivery and six days later: The concentration of 17-hydroxycorticosteroids in the plasma of women just after delivery was found to be 36.0 ± 2.7 μgm. per 100 ml., and six days after delivery it was 1.99 ± 0.51 μgm. per 100 ml. (Fig. 1).

DISCUSSION

The level of 17-hydroxycorticosteroids in the blood of pregnant women in the last trimester, and especially after delivery, is very high. In the blood of patients under stress due to surgical operations or traumatic shock, similar levels of 17-hydroxycorticosteroids have been found (7). Pregnancy and delivery may be looked upon as a form of stress causing stimulation of the adrenal cortex.

Animal experiments show that the administration of estrogens has a stimulating effect on the adrenal glands mediated by the pituitary (ACTH) (8). It is a well known fact that in late pregnancy a large amount of estrogen is circulating in the blood and may cause adrenal stimulation. According to current concepts, the production and excretion of estrogens seem to increase steadily during pregnancy. This increased estrogen production may induce the increasing levels of 17-hydroxycorticosteroids in the blood.

It has been established that during the last trimester there is a rapid growth of the fetal adrenals, and subsequent degeneration, beginning at or just previous to birth. The fetal cortex may therefore be contributing to the increased level of the 17-hydroxycorticosteroids in the blood during the latter part of pregnancy. This may account for part of the increased concentration of 17-hydroxycorticosteroids in the latter part of pregnancy, but does not explain the increase in early pregnancy, nor the very high values just after delivery.

SUMMARY

During pregnancy there is an increase in the blood levels of 17-hydroxycorticosteroids as measured by the method of Nelson and Samuels (6). A positive correlation between the level of these steroids and the duration

of pregnancy was found. The concentration of 17-hydroxycorticosteroids in the blood during the last trimester increased to about four times the normal value; immediately after delivery it increased to about seven times the normal value; but by the sixth day after delivery it had dropped to approximately the normal level.

Acknowledgment

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NEUTRAL STEROIDS IN THE URINE OF A PATIENT WITH A LUTEOMA OF THE OVARY*

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ALTHOUGH studies have been made of the urinary excretion of steroids in patients suffering from adrenal cortical hyperplasia and adrenal cortical carcinoma, no systematic investigation has been made of the urine of a patient with luteoma of the ovary. This report is based on the analysis of two collections of urine from a woman with this rare disease. One sample consisted of 19 liters which was worked up by Engel, and a second sample of 53 liters which was studied by Dorfman.

DESCRIPTION OF PATIENT

Initial history: The patient was a 21-year-old colored female whose chief complaint was amenorrhea of five years' duration. Menses had appeared at the age of 12 and had recurred at intervals of twenty-eight to thirty days, lasting six to seven days. At the age of 16 her menses suddenly ceased. Six months later she noted that her voice was lower pitched. Her weight decreased from 125 to 115 pounds. At the age of 18 hair began to appear on her cheeks and upper lip in increasing amounts, until at the age of 20 she began to shave. On admission, she admitted to shaving almost every day. At the age of 20 she noted that her clitoris was enlarging. An active sexual life, begun at the age of 13, was maintained. She had noticed shortly before admission that orgasm was not as frequent as previously, and that it took her a longer time to achieve it. Libido remained heterosexual. Her breasts had become somewhat smaller.

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* This work was essentially completed during the years 1940–1941. Changes of activities during the war period and the geographic dispersion of the authors have prevented publication until this time.

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Surgery: A right salpingo-oophorectomy was carried out. The tumor measured 8×6×6 cm. The cut surface was orange-yellow in color. The consensus, after microscopic study by various gynecological pathologists, was that the tumor was a luteoma of the ovary.

Postoperative course: The immediate postoperative period was uneventful. She menstruated twenty-seven days after surgery. Two months later she observed that she needed to shave but three times in two weeks.

Six months after surgery, the menses continued to recur regularly with moderate pain. Facial acne had disappeared and the hair on her cheeks and lips was very sparse and thin, requiring shaving about every two or three weeks. Frontal alopecia was definitely diminished. The breasts were fuller and slightly larger. The clitoris was still enlarged, although she stated that she seemed to enjoy her sex life more adequately since surgery. The voice, although still low pitched, seemed definitely higher.

HORMONE ASSAYS

A number of assays were performed. On January 4, 1940, 17-ketosteroids were found to be 79 mg. per twenty-four hours. A urine sample collected from March 27 to 30, 1940, assayed 74 mg. of 17-ketosteroids, 128 I.U. of androgens, and 200 I.U. of estrogens per twenty-four hours.

Study A (Engel)

The urine (19 liters) was collected by the patient and stored under a layer of toluene.

Hydrolysis and extraction: The urine was heated to boiling in 1,100-ml. portions, acidified with 110 ml. of concentrated HCl and boiled for fifteen minutes. The cooled urine was transferred to a Hershberg-Wolfe extractor (1, 2) and extracted with carbon tetrachloride for one hour. The solvent was evaporated off on the steam bath.

Separation of neutral and acidic fractions: The residues from carbon tetrachloride extracts representing 5,500 ml. of urine were combined and dissolved in 250 ml. of ether. The ether solution was washed successively with 25-ml. portions of N sodium bicarbonate, 2N sodium hydroxide, 1 per cent sodium hydrosulfite in 2N sodium hydroxide, and water. The ether solution was dried over sodium sulfate and evaporated to dryness. The total residue consisted of 3.2 Gm. of syrup.

Fractionation of the neutral fraction: Ketonic material was separated from the nonketonic by means of Girard reagent P. Both fractions were re-treated with Girard reagent P and 1.5 Gm. of ketonic and 0.88 Gm. of nonketonic material were obtained.

Treatment of both fractions with succinic anhydride yielded 588 mg. of nonketonic alcohols, 1.72 Gm. of ketonic alcohols, 159 mg. of nonalcoholic ketones and 186 mg. of nonketonic nonalcoholic material.

The two alcoholic fractions were separated further by precipitation with digitonin, yielding 174 mg. of nonketonic-3β-alcohols, 301 mg. of

nonketonic alcohols not precipitated by digitonin, 489 mg. of ketonic-3β alcohols and 385 mg. of ketonic alcohols not precipitated by digitonin.

Chromatographic analysis of the neutral non-alcoholic ketone fraction: The neutral nonalcoholic ketone fraction (159 mg.) was dissolved in 5 ml. of benzene and adsorbed on a column of 5 Gm. of aluminum oxide (Merck, according to Brockmann). Elution with successive portions of pentane, pentane-benzene mixtures, benzene, acetone and ethanol yielded forty-nine fractions from which no crystalline products could be isolated.

Chromatographic analysis of the nonketonic-3β-alcohols: The nonketonic-3β-alcohols precipitated by digitonin (174 mg.) were dissolved in 9 ml. of benzene and adsorbed on a column of 6 Gm. of aluminum oxide. Elution was carried out with 10-ml. portions of solvents, each solvent being used until no residue was obtained upon evaporation of the eluate. These solvents were pentane, mixtures of pentane and benzene, benzene, mixtures of benzene and acetone, acetone, and finally absolute ethanol.

Only the material eluted by benzene containing 1 per cent acetone yielded a crystalline residue. After two re-crystallizations from aqueous ethanol, 9 mg. of a compound with a melting point[1] of 144–145° were obtained. This material was shown by infrared analysis[2] to be Δ^{16}-androstene-3α-ol.

Chromatographic analysis of the neutral nonketonic alcohols not precipitated by digitonin: The neutral nonketonic alcohols not precipitated by digitonin (300 mg.) were dissolved in 5 ml. of benzene and adsorbed on a column of 9 Gm. of aluminum oxide. Elution was carried out with the following solvents: benzene (50 ml.), benzene containing 1 per cent acetone (70 ml.), benzene containing 5 per cent acetone (50 ml.), benzene containing 10 per cent acetone (30 ml.), 1:1 benzene-acetone (5 ml.), acetone (45 ml.), ether (50 ml.) and absolute ethanol (100 ml.).

The fraction eluted by acetone crystallized upon rubbing with 95 per cent ethanol. After four re-crystallizations from 95 per cent ethanol and acetone-methanol mixtures, 8 mg. of material with a melting point of 228.5–229.5° were obtained. This product gave no depression upon admixture with pregnane-3α,20α-diol.

Chromatographic analysis of the neutral alcoholic ketones not precipitated by digitonin: The neutral alcoholic ketones not precipitated by digitonin (385 mg.) were dissolved in 10 ml. of carbon tetrachloride and adsorbed on a column of 12 Gm. of aluminum oxide. Elution was carried out as indicated in Table 1.

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[1] All melting points in Study A are corrected.

[2] We are greatly indebted to the late Dr. Konrad Dobriner for performing the infrared analysis and identification.

TABLE 1. CHROMATOGRAPHIC ANALYSIS OF THE KETONIC ALCOHOLS NOT PRECIPITATED BY DIGITONIN

Fraction No.	Solvent	Vol. (ml.)	Product
1–4	CCl_4	80	—
5–9	0.1% Ethanol in CCl_4	80	—
10–14	0.2% EtOH in CCl_4	90	—
15	0.3% EtOH in CCl_4	10	Crystals, m.p. 168.5–169.5°
16	0.3% EtOH in CCl_4	20	Crystals, m.p. 164.5–169.5°
17	0.3% EtOH in CCl_4	20	Syrup
18	0.3% EtOH in CCl_4	20	Crystals, m.p. 126.5–129.5°
19	0.3% EtOH in CCl_4	20	Syrup
20	0.3% EtOH in CCl_4	20	Syrup
21	0.3% EtOH in CCl_4	20	Syrup
22–24	0.5% EtOH in CCl_4	50	Syrup
25–27	1% EtOH in CCl_4	60	Syrup
28–29	2% EtOH in CCl_4	40	Syrup
30–32	5% EtOH in CCl_4	60	—

Fractions 15 and 16 were combined. After re-crystallization from aqueous methanol, 32 mg. of androsterone (m.p. 180.5–182°) were obtained. No depression of the melting point was noted when this material was mixed with androsterone. Acetylation of this product by warming with acetic anhydride and pyridine yielded androsterone acetate which, after re-crystallization from methanol, melted at 162.5–163° and showed no depression of the melting point upon admixture with an authentic specimen of androsterone acetate.

Fractions 17–20 were benzoylated by warming with benzoyl chloride and pyridine. After precipitation with water the products were re-crystallized from methanol. Fraction 17 yielded 11 mg. of material with a melting point of 134–136°. Fraction 18 yielded 29 mg. of a compound (m.p. 157.5–158°) which was not depressed upon admixture with etiocholane-3α-ol-17-one benzoate. Fraction 19 yielded 36 mg. of etiocholanolone benzoate (m.p. 162–163°). Fraction 20 yielded 6 mg. of etiocholanolone benzoate.

Upon saponification of 25 mg. of benzoate with aqueous methanolic NaOH, 10 mg. of etiocholane-3α-ol-17-one (m.p. 147–149°) were obtained. No depression in melting point was noted when this material was mixed with an authentic specimen of etiocholanolone.

Digitonin precipitation of the neutral ketonic-3β-alcohols: The neutral ketonic alcohols precipitated by digitonin were re-treated with digitonin in 60 per cent methanol in the manner previously described. After cleavage of the digitonides with pyridine and removal of digitonin, 216 mg. of

ketonic 3β-alcohols and 170 mg. of ketonic alcohols not precipitated by digitonin were obtained.

The fraction not precipitated by digitonin yielded 59 mg. of androsterone (m.p. 178.5–179.5°) after two re-crystallizations from aqueous methanol, and 64 mg. of material having a somewhat lower melting point. The low-melting material, upon treatment with benzoyl chloride and pyridine and re-crystallization of the product from aqueous methanol, yielded a compound (m.p. 171–172°) which gave no depression upon admixture with an authentic specimen of androsterone benzoate.

The material precipitated by digitonin yielded 53 mg. of a compound (m.p. 178–180°) which gave no depression upon admixture with an authentic specimen of androsterone and which depressed the melting point of isoandrosterone. Ninety-five milligrams of lower-melting material were obtained. This substance was benzoylated and the crude product, after re-crystallization from aqueous methanol, melted at 169° and gave no depression when mixed with androsterone benzoate.

Study B (Dorfman)

Fifty-three liters of urine, representing specimens over a period of about 31 days, were collected under toluene. The urine was acidified with hydrochloric acid, 10 cc. per 100 cc. of urine, and hydrolysis and extraction were carried out simultaneously with benzene; the neutral compounds were then separated as described by Fish and Dorfman (3).

The total neutral fraction amounted to 9.8 grams. It was separated into the following fractions: ketonic alcohols not precipitated by digitonin, ketonic-3β-alcohols, nonalcoholic ketones, nonketonic alcohols, and nonketonic nonalcohols. The separations were made with Girard's reagent T (4) for the ketones, succinic acid anhydride (5) for the alcoholic compounds, and digitonin for the 3β-hydroxy compounds (3). The ketonic-3β-alcoholic, the ketonic alcoholic not precipitated with digitonin, and the nonalcoholic ketonic fractions yielded crystalline steroids.

Isolation of dehydroepiandrosterone: The ketonic-3β-alcohol fraction yielded, after re-crystallization from aqueous ethanol and methanol, 8 mg. of a compound melting at 145–146°. When mixed with an authentic sample of dehydroepiandrosterone (m.p. 146–148°) a melting point of 146–148° was found. Acetylation yielded a compound with a melting point of 165–166°. When mixed with dehydroepiandrosterone acetate the melting point was not depressed.

Isolation of androsterone and etiocholane-3α-ol-17-one: The ketonic alcohols not precipitated with digitonin were dissolved in 70 cc. of carbon tetrachloride and adsorbed on 22 grams of aluminum oxide. Elutions were carried out with 50-cc. portions of carbon tetrachloride and carbon tetra-

chloride containing 0.1 to 2 per cent of absolute ethanol. From one fraction eluted with carbon tetrachloride and two fractions eluted with 0.1 per cent ethanol in carbon tetrachloride, crystalline fractions were obtained. The yields were 7 mg. (m.p. 176–179°)[3], 80 mg. (m.p. 179–180°) and 18 mg. (m.p. 178–179°). No depressions in melting points were noted when mixed with an authentic sample of androsterone. A sample of the 80-mg. crop was acetylated and yielded an acetate with a melting point of 158–160° which, when mixed with an authentic sample of androsterone acetate (m.p. 159–160°), melted at 158–160°. The total yield of androsterone was 105 mg. Three eluates (0.2 and 0.3 per cent ethanol in carbon tetrachloride) were combined and, after four re-crystallizations from aqueous methanol, yielded 93 mg. of etiocholane-3α-ol-17-one (m.p. 140°–143°). No depression was found with an authentic sample (m.p. 142–144°). The benzoate was made, which melted at 156–158°.

Isolation of $\Delta^{2 \text{ or } 3}$*-androstene-17-one:* The nonalcoholic ketones (1.0 gram) were dissolved in 50 cc. of carbon tetrachloride and adsorbed on 15 grams of aluminum oxide. The first four elutions (50 cc. of carbon tetrachloride) yielded a highly viscous residue. This residue was sublimed at 1×10^{-4} microns at 100°C. for $1\frac{1}{2}$ hours. The sublimate was re-crystallized from 80 per cent methanol and yielded 153 mg. of a compound which softened at 63° and melted at 92–95°. The material was twice crystallized from absolute methanol to yield 107 mg. of a compound which melted at 95–97°. The compound gave a positive Zimmermann reaction and formed no acetate by the usual method. The compound was mixed with an authentic sample of $\Delta^{2 \text{ or } 3}$-androsterone-17 (m.p. 95–98°) and the melting point of the mixture was 96–98°.

DISCUSSION

Table 2 is a summary of the steroids isolated from the urine of a patient with a luteoma of the ovary. Of special significance is the increased concentration of androsterone. In Study A, 16 mg. per liter of androsterone was isolated, whereas in Study B, 2.0 mg. of androsterone per liter plus 2.0 mg. of $\Delta^{2 \text{ or } 3}$-androsterone-17 (derived from androsterone) was isolated. Of further interest is the fact that the concentration of dehydroepiandrosterone was normal. The concentration of etiocholane-3α-ol-17-one may have been increased somewhat. Callow and Callow (6) have isolated androsterone (1.3 mg. per liter), etiocholanol-3(α)-one-17 (1.3 mg. per liter) and dehydroepiandrosterone (0.2 mg. per liter) from the urine of normal women.

[3] All melting points in Study B were taken with the Fischer-Johns apparatus and are uncorrected.

TABLE 2. COMPOUNDS ISOLATED FROM URINE OF PATIENT WITH A LUTEOMA OF THE OVARY

Compound	Study A (mg./liter)	Study B (mg./liter)
Androsterone	16	2.0
$\Delta^{2 \text{ or } 3}$-Androstene-17-one	—	2.9
Etiocholane-3α-ol-17-one	2.7	1.8
Δ^{16}-Androstene-3α-ol	0.47	—
Dehydroepiandrosterone	—	0.15
Pregnane-3α,20α-diol	0.4	—

Similar patterns of androsterone and dehydroepiandrosterone concentrations in urine have been reported in patients with adrenal hyperplasia (7, 8, 9). In adrenal cancer the concentration of dehydroepiandrosterone is often enormously increased (10, 11), whereas the concentration of androsterone may or may not be increased (2, 11).

The occurrence of Δ^{16}-androstene-3α-ol in the urine of this patient is noteworthy. This compound has been isolated from the urine of normal males (12) and from the urine of women with adrenal cortical tumors (13). Its appearance in the urine of a patient with an ovarian luteoma suggests that it may originate in the ovary as well as in the adrenal cortex.

SUMMARY

The urinary neutral steroids have been studied in a 21-year-old woman with a luteoma of the ovary. Androsterone, etiocholane-3α-ol-17-one, Δ^{16}-androstene-3α-ol, dehydroepiandrosterone, and pregnane-3α,20 α-diol were isolated and identified. The androsterone content of the urine was distinctly increased. The increase in etiocholane-3α-ol-17-one concentration was not convincing.

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CHEMICAL METHOD FOR THE DETERMINATION OF 17-HYDROXYCORTICOSTEROIDS AND 17-KETOSTEROIDS IN URINE FOLLOWING HYDROLYSIS WITH β-GLUCURONIDASE*

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METHODS for the estimation of metabolites of the adrenocortical steroids thus far published have given only partially satisfactory estimates of adrenocortical function. A method has been developed which gives reproducible determinations of 17,21-dihydroxy-20-ketones and 17-ketosteroids present in urine, either in the free form or conjugated as glucuronides.

The acid hydrolysis of urinary adrenal steroid metabolites has been accomplished with varying degrees of success by a number of investigators (1–4), but there is considerable evidence suggesting loss or alteration of steroids during the hydrolytic process (5, 6). It has been shown that a large part of the adrenocortical steroids in urine are conjugated as glucuronides (7–10). In the present method hydrolysis with β-glucuronidase was employed and consistent levels of adrenocortical steroid metabolites were found after chromatography on a Florisil column.

The phenylhydrazine-sulfuric acid reaction of Porter and Silber (11) for 17-hydroxycorticosteroids, successfully used by Nelson and Samuels (12) for the quantitative determination of these compounds in blood, has been applied to the chromatographed fractions from urine.

MATERIALS AND METHODS

Materials: Materials for the phenylhydrazine-sulfuric acid test for 17-hydroxycor-

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ticosteroids are those given by Nelson and Samuels (12). The following reagents are also required:

1. Penicillin solution, containing 1,000,000 units in 10 ml. of physiologic saline.
2. Sodium acetate (0.2 M) and acetic acid (0.2 M) solutions for buffer system.
3. Standardized solution of spleen β-glucuronidase[1] containing 5,000 Fishman units/ml.
4. Recently redistilled chloroform.
5. NaOH (0.1 N).
6. HCl (0.1 N).
7. Anhydrous Na_2SO_4.

The materials for the 17-ketosteroid reaction are those described by Callow *et al.* (13).

Method: To a 30-ml. aliquot of a freshly collected 24-hour urine specimen, 2.0 ml. each of the acetic acid and sodium acetate solutions are added. The sample is then adjusted to pH 4.5 by further additions of acetic acid if necessary. To this solution 25,000 units of penicillin and 10,000 Fishman units of β-glucuronidase solution are added with thorough mixing. The urine is then incubated either at 37.5° C. for twenty-eight hours or at 47° C. for fifteen hours. The latter procedure gives slightly higher values.

The reaction mixture is removed from the incubator and immediately extracted three times with 15-ml. aliquots of chloroform. The extraction is carried out in a 100-ml. centrifuge tube by mixing with a footed stirring rod. Following each extraction the contents of the tube are centrifuged in order to resolve the emulsion formed, and the chloroform layer is removed by means of a serum-lifter.[2] The three chloroform extracts are transferred to another 100-ml. centrifuge tube.

The combined extracts are then washed twice with 5.0 ml. of 0.1 N NaOH and once with 5.0 ml. of 0.1 N HCl. Each of these washes is individually back-extracted with an equal amount of chloroform. Approximately 3.0 Gm. of anhydrous sodium sulfate are added to the combined chloroform extracts and allowed to stand for a few minutes. The chloroform layer is then transferred to a 125-ml. Erlenmeyer flask by filtering through Whatman No. 1 filter paper. The sodium sulfate is washed with two 10-ml. aliquots of chloroform and added to the original extract. The contents of the flask are dried under a gentle stream of air in a water-bath maintained at a temperature below 50° C.

The dry residue is dissolved in 5.0 ml. of chloroform and transferred quantitatively to the Florisil column prepared according to the method of Nelson and Samuels (12). The column is eluted first with 25 ml. of chloroform and subsequently with 25 ml. of 4 per cent methanol-chloroform. These combined eluates are collected in a 125-ml. Erlenmeyer flask and dried under a gentle stream of air. This fraction contains the free and glucuronide-conjugated 17-ketosteroids from the urine. The column is next eluted with 45 ml. of 25 per cent methanol in chloroform and the eluate is collected in a 50-ml. round-bottom flask. The contents of the flask are dried in the same manner as previously described. This fraction contains the 17-hydroxycorticosteroid fraction. Care should be taken that the column never runs dry during the entire elution.

The 17-hydroxycorticosteroid fraction is dissolved in 4.0 ml. of absolute methanol and 1.0-ml. or 2.0-ml. aliquots are transferred to test tubes for the phenylhydrazine-sulfuric acid reaction. Equal aliquots are transferred to another set of test tubes for blank determinations with sulfuric acid alone. The 1.0-ml. portions are diluted to 2.0 ml. with absolute methanol. To the first set of tubes 3 ml. of phenylhydrazine-sulfuric acid re-

[1] Obtained from the Warner Institute of Therapeutic Research, New York, N. Y.

[2] Supplied by the Zoo-Line Apparatus Co., Salt Lake City, Utah.

agent are added, and 3 ml. of the sulfuric acid alone are introduced into the second set. All tubes are transferred to a 60° C. water-bath and allowed to remain for sixty minutes, after which time they are cooled for five minutes under running tap water. The solutions are read on a Beckman model DU spectrophotometer in 3-ml. cuvettes at 370, 410 and 450 mμ.

For the calculation of quantities of steroid present, the sulfuric acid blank is subtracted from the readings given by the phenylhydrazine-sulfuric acid reaction. The sum of the corrected readings at 370 mμ and 450 mμ is subtracted from the product of 2× the corrected reading at 410 mμ and calculated from a standard curve of crystalline hydrocortisone[3] covering the range between 25 and 100 μg. (14).

The 17-ketosteroid fraction is redissolved in 3.0 ml. of absolute ethanol and 1.0-ml. or 0.5-ml. aliquots are transferred to test tubes and dried under a gentle stream of air. The dry residue is dissolved in 0.2 ml. of absolute ethanol and the Callow modification (13) of the Zimmermann reaction is carried out. After the reaction is complete, the samples are diluted with 3.0 ml. of absolute ethanol and read on the Beckman spectrophotometer at 520 mμ. The value is calculated from a standard calibration curve of androsterone (25–150 μg.).

RESULTS

The results of variations in the concentration of β-glucuronidase are presented in Figure 1. The incubation period was twenty-eight hours and temperature 37.5° C. From these data 10,000 Fishman units of β-glucuronidase per 30 ml. of urine was chosen as the most satisfactory concen-

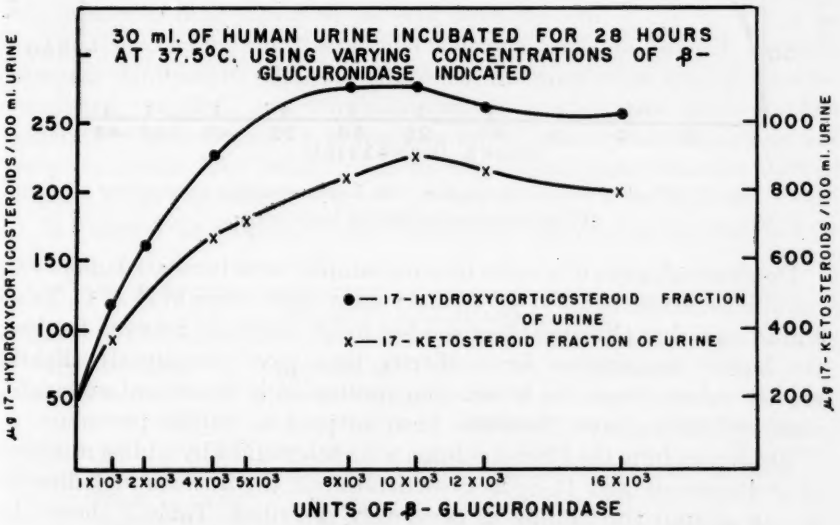

FIG. 1. Effect of concentration of β-glucuronidase on recovery of 17-hydroxycorticosteroids from urine.

[3] Generously supplied by Drs. J. M. Carlisle and E. Alpert, Merck and Co., Rahway, N. J.

tration. The effect of incubating urine with 10,000 units of β-glucuronidase at 37.5° C. for varying periods of time is given in Figure 2. The figure suggests that the ideal time of incubation at 37.5° C. is twenty-eight hours for both the 17-ketosteroid and the 17-hydroxycorticosteroid fractions. These results closely approximate those of Cohen (10).

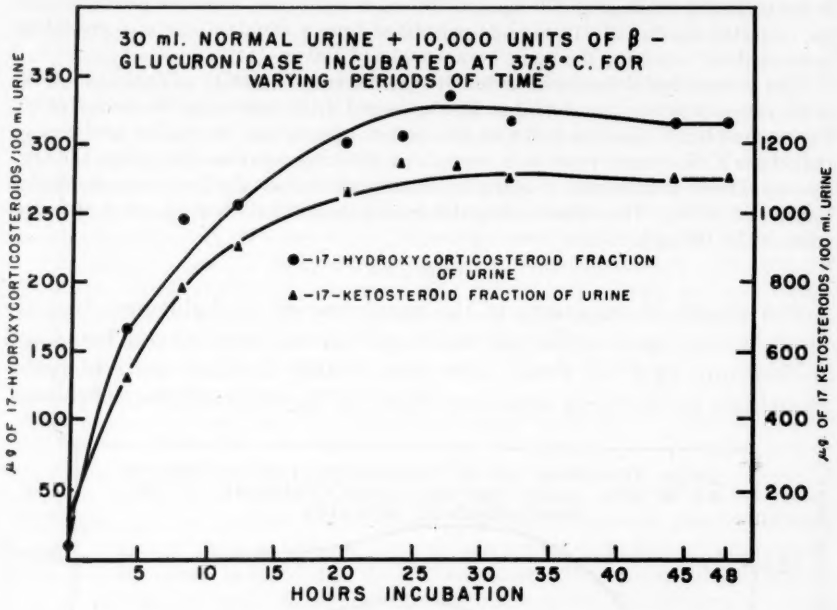

FIG. 2. Effect of time of incubation with β-glucuronidase on recovery of 17-hydroxycorticosteroids from urine.

Duplicate aliquots of a series of urine samples were incubated under two conditions: fifteen hours at 47° C. and twenty-eight hours at 37.5° C. Table 1 illustrates that although there was not much difference between the two, the higher temperature for a shorter time gave consistently slightly greater values. Since the fifteen-hour incubation is convenient overnight, these conditions have, therefore, been adopted as routine procedure.

Recoveries from the Florisil column were determined by adding mixtures of androsterone and 17-hydroxycorticosterone (hydrocortisone) directly to the column and eluting as previously described. Table 2 shows the results obtained by this procedure. The quantities of the two compounds added to the column are approximately the amounts present in 30 ml. of normal urine.

Table 3 demonstrates the recoveries of mixtures of androsterone and

TABLE 1. INFLUENCE OF INCUBATION WITH β-GLUCURONIDASE FOR SHORTER TIME AT HIGHER TEMPERATURE ON YIELD OF 17-HYDROXYCORTICOSTEROIDS AND 17-KETOSTEROIDS

(10,000 Fishman units of spleen glucuronidase at pH 4.5–4.8)

Sample	28 hrs. at 37° C.		15 hrs. at 47° C.	
	17,21-OH steroids μg./ml.	17-Keto-steroids μg./ml.	17,21-OH steroids μg./ml.	17-Keto-steroids μg./ml.
C 1st day	6.5	9.3	8.1	10.8
C 2nd day	7.0	9.5	8.8	10.6
C 3rd day	7.0	9.5	7.4	11.1
C 4th day	6.6	9.0	7.6	10.5
C 5th day	6.6	9.3	7.5	10.8
C 6th day	6.6	10.0	8.0	11.1
L	16.5	10.6	18.0	8.4
P	2.25	3.1	2.73	3.3

hydrocortisone added to urine following β-glucuronidase hydrolysis. When hydrocortisone was added to urine prior to the incubation period the recoveries ranged from 81 per cent to 98 per cent.

The reproducibility of the method is demonstrated in Table 4. In every instance duplicate or triplicate samples of the same urine were incubated, extracted, purified, and chromatographed in the same manner. The phenylhydrazine-sulfuric acid reaction for 17-hydroxycorticosteroids was very consistent. The values given by the Zimmermann reaction showed slightly wider variation.

In Figure 3 the absorption spectrum for the color formed by the phenyl-

TABLE 2. RECOVERY OF MIXTURES OF HYDROCORTISONE AND ANDROSTERONE FROM THE FLORISIL COLUMN

Hydrocortisone added to column (μg.)	Androsterone added to column (μg.)	Recovery	
		Hydrocortisone %	Androsterone %
150.0	181.0	104.0	107.0
125.0	149.0	95.0	99.0
100.0	109.0	94.0	105.0
75.0	72.0	86.0	103.0
50.0	54.0	101.0	103.0
25.0	36.0	101.0	98.0

TABLE 3. RECOVERY OF HYDROCORTISONE AND ANDROSTERONE ADDED TO URINE FOLLOWING β-GLUCURONIDASE HYDROLYSIS

Urine sample	Material added to urine		Recovery	
	Hydrocortisone (μg.)	Androsterone (μg.)	Hydrocortisone %	Androsterone %
36	108.0	150.0	93.0	98.0
37	91.0	100.0	94.0	106.0
38	73.0	75.0	90.0	90.0
39	54.0	50.0	92.0	93.0
40	36.0	25.0	96.0	95.0

hydrazine-sulfuric acid reaction with crystalline hydrocortisone is compared with the spectrum of the color obtained with the 17-hydroxycorticosteroid fraction of urine extracts obtained as described. Both show the characteristic peak at 410 mμ. Figure 4 illustrates the spectra obtained following the application of the Zimmermann reaction to crystalline androsterone and to the 17-ketosteroid fraction of urine extracts. The characteristic peak at 520 mμ is demonstrated for both preparations.

The Zimmermann reaction on the 25 per cent methanol fraction failed to give the characteristic color for 17-ketosteroids. When the phenylhydrazine-sulfuric acid reaction was run on the 4 per cent methanol fraction, no 17-hydroxycorticosteroids were found. The chromatographic procedure thus shows a good separation of the two types of compounds. The application of sulfuric acid to urine extracts always poses the question of background color, but the magnitude of the sulfuric acid blank in this procedure after partitioning and chromatography was found to be negligible. Control blanks for the determination of 17-ketosteroids following the acid hydrolysis of urine by other methods often are of high magnitude.

TABLE 4. REPRODUCIBILITY OF METHOD ON URINE SAMPLES

(Determination on equal aliquots of urine)

Urine sample No.	17-Hydroxycorticosteroids (μg./100 ml.) Aliquot No.			17-Ketosteroids (μg./100 ml.) Aliquot No.		
	1	2	3	1	2	3
A	332.0	332.0	332.0			
B	249.0	—	249.0			
C	304.0	316.0	304.0	1161.0	1254.0	1307.0
D	415.0	396.0	396.0	1755.0	1636.0	1538.0

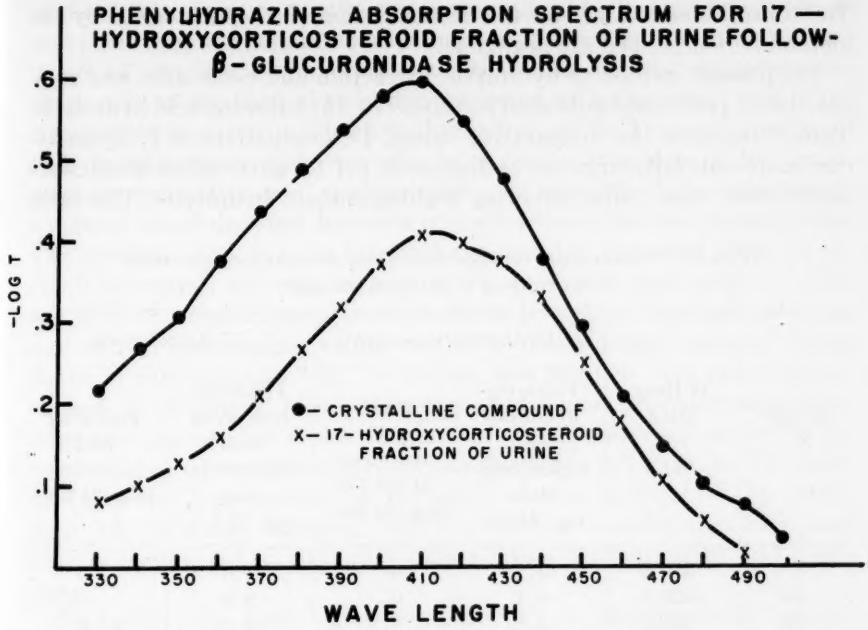

FIG. 3. Absorption spectrum of color produced by reaction of phenylhydrazine with urine extract, compared with that after reaction with hydrocortisone (compound F).

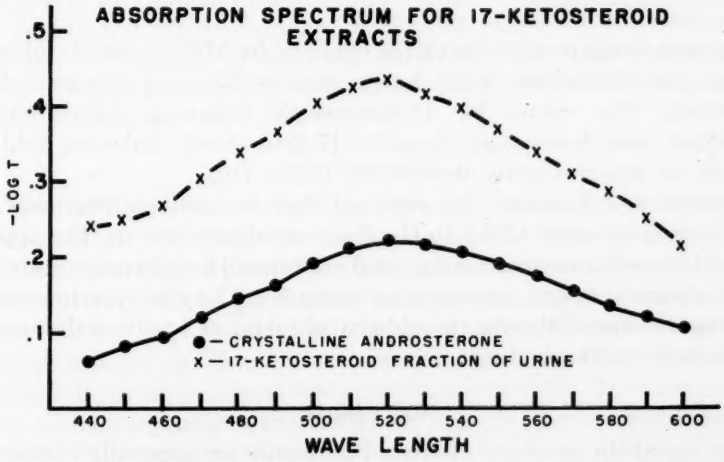

FIG. 4. Absorption spectrum of color produced by the Zimmermann reaction with urine extract, compared with that produced by the reaction with androsterone.

The control-blank values for the determination of 17-ketosteroids by the method here described are negligible.

The present method of hydrolysis, extraction and estimation was compared with previously published methods (13, 15) following acid hydrolysis. Table 5 indicates the comparative values. Determinations of 17-hydroxycorticosteroids following acid hydrolysis at pH 1.0 gave values which were much lower than those following β-glucuronidase hydrolysis. The table

TABLE 5. COMPARISON OF PROPOSED METHOD OF HYDROLYSIS WITH PREVIOUSLY PUBLISHED METHODS

Sample No.	24-Hour urine vol. (ml.)	17-Hydroxycorticosteroids		17-Ketosteroids	
		Following hydrolysis with β-glucuronidase (mg./24 hrs.)	Following acid hydrolysis at pH 1.0 (mg./24 hrs.)	Following hydrolysis with β-glucuronidase (mg./24 hrs.)	Following acid hydrolysis (mg./24 hrs.)
47	650.0	3.2	0	1.0	2.6
48	1020.0	6.1	0	8.6	—
49	825.0	3.1	0.2	4.3	11.0
50	418.0	3.2	0.1	1.6	7.3
51	1160.0	4.8	0.3	2.0	3.0
52	600.0	5.9	0.3	5.2	19.6
69	1360.0	4.0	0.2	2.6	7.0

also gives a comparison of the values obtained for 17-ketosteroids following strong acid hydrolysis, with determinations following β-glucuronidase hydrolysis. The values for 17-ketosteroids following β-glucuronidase hydrolysis were lower than those for 17-ketosteroids following acid hydrolysis, as was previously observed by Cohen (10).

Bernfeld and Fishman (16) reported that desoxyribonucleic acid acts as a co-enzyme when added to the β-glucuronidase system. The application of this technique to urine has been attempted, but of twelve individual urine specimens tested, none gave an increase in 17-hydroxycorticosteroids or 17-ketosteroids following the addition of 1.0 ml. of 3 per cent desoxyribonucleic acid to 30 ml. of urine.

DISCUSSION

The metabolic products of steroid hormones are generally excreted as conjugates. In the case of the cortical steroids the instability of the more

oxidized side chain makes the use of strong acid hydrolysis unfeasible. It is evident from the results reported here, as well as those of other workers (9, 10, 17, 18), that acid hydrolysis at pH 1 either does not release the major portion of the cortical steroids conjugated with glucuronic acid or destroys the characteristic side chain during hydrolysis. The real excretion of 17-hydroxycorticosteroids can only be evaluated, therefore, if the conjugates are completely split, as glucuronides are split with β-glucuronidase, in a manner which does not destroy a major fraction of the steroid molecules. The problem, then, is to be sure that the products measured after the enzymic hydrolysis are really steroids of this type. The applications of color reactions to urine extracts purified simply by solvent partition methods lead to high and irregular values even in Addison's disease, because of nonsteroidal contaminants (18). As Nelson and Samuels (12) have demonstrated on extracts of blood, their chromatographic procedure eliminates large amounts of nonsteroidal material. Patients with no functioning adrenal cortical tissue did not give measurable levels in the blood. The same condition has been observed in the urines of such patients by this procedure (19). It seems, therefore, that this method measures one important form of conjugated cortical steroids with reasonable accuracy and specificity.

Conversely, the lower values obtained for the 17-ketosteroid fraction suggest that the glucuronides are only one part of the total 17-ketosteroids recovered after strong acid hydrolysis. This confirms the work of Bitman and Cohen (20). Whether glucuronides represent a physiologically distinct fraction or whether their level is simply dependent on the relative activities of the various conjugating systems, remains to be determined. Although the value of determining steroids in the urine conjugated as glucuronides is apparent, the conjugation of steroid metabolites as sulfates or other esters may also be important.

SUMMARY

A method has been described for the quantitative separation and determination of 17-hydroxycorticosteroids and 17-ketosteroids in urine following enzymatic hydrolysis with β-glucuronidase. Extraction with chloroform and partial purification with sodium hydroxide and sodium sulfate followed by chromatographic separation on the Florisil column gave fractions with no significant contamination by nonsteroidal components. The phenylhydrazine-sulfuric acid reaction was used for the determination of the 17-hydroxycorticosteroid fraction and the Zimmermann reaction for the 17-ketosteroid fraction.

This method appears to give a better separation and is more specific

for the determination of the two different fractions than other methods previously described. Only part of the 17-ketosteroids, however, are freed by β-glucuronidase.

Acknowledgment

The authors wish to thank Miss Carma Darley for technical assistance.

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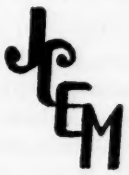

DETERMINATION OF 17-KETOSTEROIDS IN URINE

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WITHIN the past few years increasing attention has been paid to the excretion of 17-ketosteroids as a diagnostic aid. In general, the methods proposed have been time-consuming and impractical for average hospital laboratory routine. There is a need for a simple and accurate procedure capable of yielding reproducible results in the hands of the average clinical laboratory technician.

Such a method was proposed by Drekter *et al.* in 1952 (1). However, in duplication of this method, the authors found that the final color was markedly unstable, fading as much as 5 to 10 per cent in transmission readings in the first three to four minutes. In addition, recovery of added steroid varied from 20 to 150 per cent.

Studies were undertaken in an attempt to increase the stability of the color, and to afford greater accuracy in recovery of added steroid and replicate determinations. It was found that, by substituting Methyl Cellosolve (ethylene glycol monomethyl ether, Carbide and Carbon Chemicals Division of the Union Carbide and Carbon Corporation) for ethyl alcohol as the final diluent, a more stable color results. The 1 per cent *m*-dinitrobenzene reagent for the Zimmermann reaction was prepared in the same solvent. The 8 N aqueous KOH recommended by Drekter was replaced by 4 N KOH in methanol. Both of these reagents were stable for over six months when stored in the refrigerator. Another departure from the Drekter procedure was to allow the Zimmermann reaction mixture to stand for sixty minutes at room temperature for full color development before diluting it with solvent. The preparation of the samples prior to the development of color followed the Drekter method.

A. Materials and equipment used

1. Pyrex bottles of 50-ml. capacity, with ground-glass stoppers.
2. Mechanical shaker.[1]
3. Beckman model B spectrophotometer.

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[1] A mechanical Burrell wrist-action shaker was used.

4. Concentrated hydrochloric acid, C.P.
5. Ethylene dichloride, purified.
6. Meta-dinitrobenzene, purified according to the method of Drekter *et al.* (2) and used as a 1 per cent solution in Methyl Cellosolve.
7. Methyl Cellosolve (ethylene glycol monomethyl ether) (Carbide and Carbon Chemicals Division, Union Carbide and Carbon Corporation, New York, N. Y.)
8. Sodium hydroxide pellets, C.P.
9. Potassium hydroxide, C.P., 4 N in methyl alcohol, checked by titration against standard acid.
10. Dehydroisoandrosterone. A standard solution of 10 gamma per cc. prepared in methyl alcohol.
11. Methyl alcohol, C.P.

B. Method

The modified procedure is as follows: Pipette 10 ml. of urine into a 50-ml. Pyrex glass-stoppered bottle. Add 3 ml. of concentrated hydrochloric acid, and place in a boiling water bath for ten minutes. Cool and add 10 ml. of ethylene dichloride. Stopper and shake for fifteen minutes with a mechanical shaker. Separate the bottom layer and filter into a dry 50-ml. Pyrex bottle. Add 20 pellets of sodium hydroxide and shake for fifteen minutes. Filter the solvent into a dry test tube through #1 filter paper. Pipette 2 ml. of the ethylene dichloride into a dry test tube and evaporate the solvent on the water bath at 100° C. A blank is prepared by substituting 10 ml. of water for urine in the above procedure. Add to the dry extract 0.4 ml. of 1 per cent *m*-dinitrobenzene in Methyl Cellosolve, followed by 0.3 ml. of 4 N potassium hydroxide in methanol. Mix well to dissolve the dried material and allow to stand for exactly sixty minutes at room temperature. Add 5 ml. of Methyl Cellosolve and mix. The colors are read in a model B spectrophotometer at 510 millimicrons with the blank at 100 per cent transmission.

Standards are prepared by drying aliquots of a standard solution of dehydroisoandrosterone in methyl alcohol in a boiling water bath. A reagent blank is prepared when reading standards. Urine blanks are prepared by following the procedure described, substituting Methyl Cellosolve for the *m*-dinitrobenzene solution. The blanks are read in the spectrophotometer at 510 millimicrons against pure Methyl Cellosolve. The results of 7 determinations are shown in Table 1, and indicate that the interference caused by urine pigments is negligible.

TABLE 1. EFFECT OF URINE BLANKS ON OPTICAL DENSITY AT 510 MILLIMICRONS

Specimen	Optical density	Mg. equivalent of 17-ketosteroid per 24-hr. urine
JS	.004	0.17
RH	.006	0.25
JH	.002	0.09
FS	.003	0.13
TR	.001	0.04
EB	.003	0.13
TM	.005	0.22

Figure 1 shows the adherence to the Beer-Lambert law. It may be seen that the relationship is constant in the range of 0 to 50 gamma of steroid. This graph was prepared by measuring the optical density of standards treated as described in the procedure. In addition, aliquots of the standard solution of dehydroisoandrosterone were added to urine samples and analyses performed. These results were plotted on the same graph and

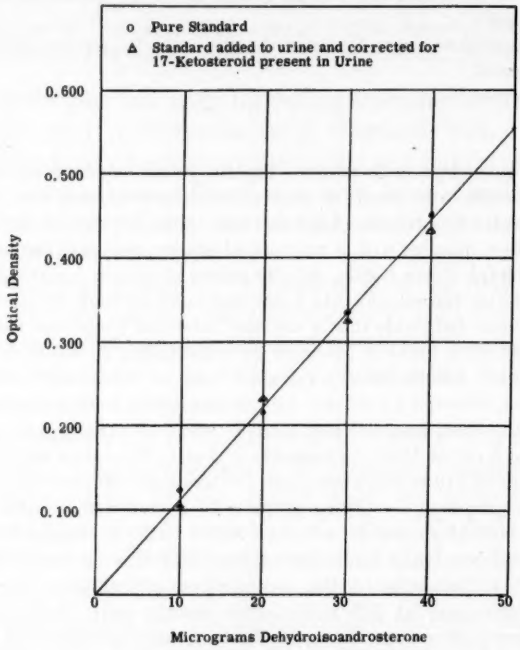

FIG. 1. Relationship between optical density and concentration of dehydroisoandrosterone.

have been corrected for 17-ketosteroids originally present in the urine. The spectral-transmission curve for dehydrosioandrosterone and for 17-ketosteroids obtained from urine is identical and is shown in Figure 2. Table 2 shows the stability of color of standards and urine extracts when treated as described in the procedure. From the table it can be seen that the color is optically stable for at least thirty minutes, and then slowly decreases in optical density. Table 3 shows the results of duplicate urine analyses. Table 4 shows the recovery of added steroid from urine samples.

Replicate analyses show excellent agreement, and recovery of added steroid varies from 96 to 104 per cent.

Further samples of urine were analyzed according to the polarographic

procedure of Barnett (3), and compared with analyses performed as described. The Barnett procedure involves the separation of 17-ketosteroids as Girard T complexes, and subsequent polarographic estimation of

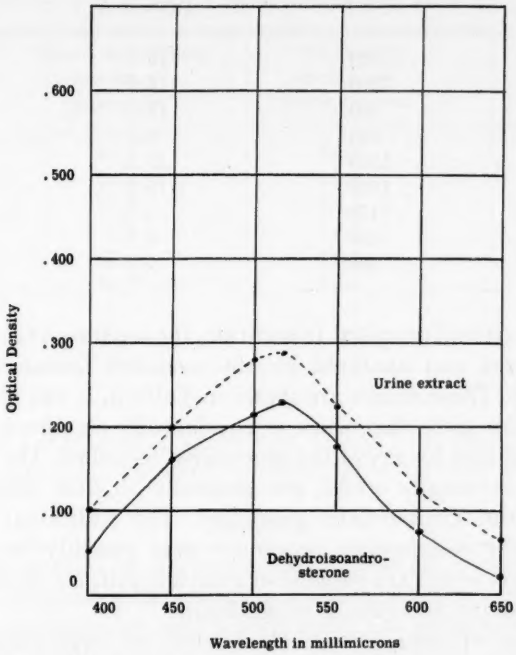

FIG. 2. Spectral curves for pure dehydroisoandrosterone and 17-ketosteroids from urine when treated by the modified Zimmermann reaction described in the procedure.

TABLE 2. STABILITY OF COLOR

Micrograms of steroid	Time in minutes after color development						
	3	5	10	20	30	40	50
	Transmission (%)						
10	78	78	78	78	78.2	78.6	79
18.3*	63	63	63	63	63.4	63.8	64.1
24.2*	54	54	54	54	54.1	54.6	55.2
25	52	52	52	52	52.1	52.4	53
28.6*	48	48	48	48	48.3	48.6	49.1
40	36	36	36	36	36.3	36.6	37
50	28	28	28	28	28.4	28.8	29.1

* Urine extracts.

TABLE 3. RESULTS OF DUPLICATE ANALYSES ON SEPARATE URINE SPECIMENS

Specimen	Urine, 24-hr. vol. (ml.)	Mg. of 17-ketosteroid	
		Analysis A	Analysis B
JS	1320	15.1	15.2
RH	1210	13.2	13.5
JH	560	14.3	14.1
FS	440	1.7	1.6
TR	1300	49.4	48.9
EB	1090	15.3	15.5
TM	1150	3.9	4.0
EB	1400	6.7	6.5
RG	500	7.1	7.2

the separated steroid complex. In addition, the separated Girard complexes were hydrolyzed and analyzed by the modified Zimmermann reaction described here. These results are shown in Table 5. It can clearly be ascertained from the table that there is no clinically significant difference in the results obtained by any of the procedures described. The polarographic procedure gives results which are generally slightly lower than those obtained by the Zimmermann procedure. The additional steroid found when using the colorimetric procedure may possibly be due to other ketonic steroids which are capable of reacting with the Zimmermann reagents. The amount, however, is insignificant.

The stability of color permits the analysis of large numbers of urines simultaneously without any special timing precautions. As there is some deviation from Beer's Law in amounts greater than 50 gamma of steroid it is advisable to repeat analyses on smaller aliquots when high concentra-

TABLE 4. RECOVERY OF DEHYDROISOANDROSTERONE ADDED TO URINE SAMPLES

Micrograms of steroid added to 10 ml. of urine	Micrograms of steroid recovered	Percentage recovery
25	24	96
25	26	102
50	51	102
50	52	104
50	51	102
75	73	97
75	74	99

TABLE 5. COMPARISON OF RESULTS OBTAINED ON URINE EXTRACTS AFTER SEPARATION OF THE GIRARD-T COMPLEX, WITH RESULTS OBTAINED USING THE PROCEDURE DESCRIBED

Specimen	17-Ketosteroids (mg./24 hrs.)		
	Girard separation, polarographic procedure	Zimmermann reaction on ketonic fraction	Modified procedure
JS	13.5	14.8	15.1
RH	11.8	13.8	13.2
JH	12.7	13.9	14.3
FS	1.4	1.5	1.7
TR	47.6	48.9	49.4
EB	13.9	14.8	15.3
TM	3.4	3.4	3.9
EB	6.3	6.4	6.7
RG	6.8	6.8	7.1

tions of steroid are present in an extract. It has been found convenient to prepare 1 ml. and 2 ml. extracts for drying prior to color development.

SUMMARY

The Drekter method for the rapid determination of urinary 17-ketosteroids has been modified in the following respects: Methyl Cellosolve is used as the diluent, *m*-dinitrobenzene is prepared in this same solvent, 4N KOH in methanol is substituted for the 8 N aqueous KOH, and the time of reaction has been changed to sixty minutes at room temperature. With these modifications, a more stable color is obtained, replicate analyses are in excellent agreement, and recovery of added steroid varies from 96 to 104 per cent. Further evidence is offered substantiating Drekter's finding that interfering pigments are almost completely removed.

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ANALYSIS OF URINARY STEROIDS FOLLOWING GLUCURONIDASE HYDROLYSIS. I. EXTRACTION AND FRACTIONATION*

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INTRODUCTION

WE have presented a preliminary description of methods for the extraction and separation of urinary corticosteroids (1,2). With the development of glucuronidase hydrolysis (3) and the consequent increased yield of corticosteroid, along with methods for paper partition chromatography (4, 5, 6), the possibility of routinely determining patterns of corticosteroids present in two- to three-day collections of human urine seemed feasible. It soon became evident that a considerable array of substances was revealed by paper chromatography of glucuronidase-hydrolyzed urines and a systematic effort at the resolution of the steroid mixture was initiated. In this paper we present the techniques evolved for the pattern analysis. The chief features of the method now routinely employed are: (a) glucuronidase hydrolysis followed by methylene chloride extraction and separation of a crude neutral lipid, (b) fractionation of the neutral lipid by silica gel chromatography into three major "steroid" eluates, and (c) paper partition chromatography of the three major "steroid" fractions, conducted so as to effect adequate separation of the component substances.

In this paper we also present data on a number of urine extracts which illustrate the separations achieved and the types of substances observed.

METHODS

Incubation and extraction of the urine: The urine is collected as two 24-hour samples, each of which is adjusted to pH 4.5 with 5 N acetic acid and incubated with 150,000 Fishman units of a crude beef-spleen glucuronidase for twenty-four hours at 37.5°C.[1] The urine is then adjusted to pH 1.0 with 12 N H_2SO_4 and placed in a Hershberg-Wolfe extractor of 1500 cc. capacity (Scientific Glass Apparatus Co.) in which the fritted glass plate has been replaced by a finely punctured platinum (0.002 in. thick)

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[1] These conditions are not optimal for maximal release of steroids but provide a quantity adequate for delineation of steroid patterns.

disc for prevention of clogging. The urine is extracted with 350 cc. of redistilled methylene chloride (dichloromethane) for eighteen hours. The methylene chloride extract is washed five times with 25–40 cc. of cold 0.1 N NaOH, five times with 25–40 cc. of distilled water and then dried with anydrous sodium sulfate (C.P.). The extract is concentrated by distillation and evaporated to dryness under a stream of nitrogen.

Preliminary silica gel chromatography: A solvent fractionation (7) of the urinary residue on silica gel (8) is useful for concentration of approximately 70 per cent by weight of nonsteroidal, pigmented material in the benzene-ether 2:1 fraction. Moreover it distributes the rather large number of urinary metabolites into three major components on the basis of polarity. This affords a separation of a number of different metabolites with similar running rates on paper.

Prior to the silica gel chromatography, an aliquot (equal to 5 per cent of the total) is removed from each of the extracts of the two 24-hour urine samples for formaldehydogenic steroid (FS) estimation (9). Extract residues remaining (95 per cent) are combined, dried under a stream of nitrogen in a warm water bath and weighed. Silica gel (Davison), 30 to 60 times the weight of the residue, is transferred to the column as a slurry with 25–50 cc. of benzene. The larger amount is employed when an excess of pigment appears to be present. The residues are then transferred to the column with benzene. The development of a typical chromatogram is outlined in Table 1. All solvents are redistilled. The ether is washed with concentrated H_2SO_4 (10) prior to distillation. The volume used for each developing solvent or solvent combination is roughly in the proportion of 10–20 cc. to 0.100 Gm. of residue weight, with the exception of benzene-ether 1:2 and ether-ethyl acetate 1:2, for each of which double the volume is employed. After the development of the chromatogram into the six designated fractions, an aliquot is removed from each for FS determination.

The benzene fraction (#1) contains a waxy material and has no FS content. The benzene-ether 2:1 (#2) removes the greater part of the urinary pigment, has very little FS and, although routinely chromatographed on paper, contains only trace amounts of urinary metabolites occurring in the subsequent fractions. The benzene-ether 1:1 (#3) and the benzene-ether 1:2 (#4) eluates are combined and constitute the "less polar" fraction (C). The ether fraction (#5) may be combined with #3 and #4. Generally it contains very little FS-producing material. The ether-ethyl acetate (#6 and #7) and the ethyl acetate (#8) eluates are combined to form the "more polar" fraction (B). The acetone (#9) and methanol (#10) fractions constitute the "most polar" fraction (A). The sum of FS values of the six fractions is generally between 70 and 140 per cent of the pre-chromatography FS value (Table 1).

Paper chromatography: The equipment and technique employed are essentially those described by Zaffaroni *et al.* (4, 5, 6) and therefore will not be outlined in detail here. Briefly, the chromatogram tanks are lined with filter paper which is wet with the developing solvent and the bottoms are covered with the same solvent to a depth of one-half inch. The troughs are always filled with 350 cc. of the appropriate solvents, such as benzene saturated with formamide or toluene saturated with propylene glycol. The tanks are allowed to equilibrate at least one hour prior to use. They are enclosed in wooden cabinets in which air is circulated by a fan and in which the temperature is maintained at 30°C.

Prior to use, all paper is washed for a week in a Soxhlet extractor with a 3:1 water-methanol solution. Three types of Whatman filter paper sheets are employed: Nos. 1, 540 and 42. Numbers 1 and 540 have similar flow rates, but the latter is cleaner and less fragile when wet. It has the slight disadvantage of stiffness. It is useful for re-

TABLE 1. DEVELOPMENT OF SILICA GEL CHROMATOGRAM*

Fraction No.	Developing solvents	Vol. of solvent (cc.)	FS (mg.)	Distribution %	
1	Benzene	25–35	0.00	0.0	
2	Benzene-ether 2:1	50	0.15	0.6	
3	Benzene-ether 1:1	50	18.27	69.2	Less polar fraction C
4	Benzene-ether 1:2	100			
5	Ether	50	0.18	0.7	
6	Ether-ethyl acetate 9:1	50			
	Ether-ethyl acetate 4:1	50			
	Ether-ethyl acetate 3:1	50			More polar
7	Ether-ethyl acetate 2:1	50	5.64	21.4	fraction B
	Ether-ethyl acetate 1:1	50			
	Ether-ethyl acetate 1:2	100			
8	Ethyl acetate	50			
9	Acetone	50	2.15	8.1	Most polar
10	Methanol	50			fraction A
		Total			
	Corrected for aliquots removed		26.39 27.69	Recovery 96% (*cf.* pre-chromatogram value of 28.86)	

* Silical gel chromatography of a 48-hour urine residue from a male, 27 years of age. The dry weight was 0.35 Gm. and the FS value 28.86 mg./48 hours.

chromatographing metabolites on which sulfuric acid chromogen spectra are to be determined. Paper #42, a coarse, porous one with a fast flow rate, is particularly helpful for separating highly hydroxylated compounds.

The paper strips are cut in 42-cm. lengths with the starting line 12 cm. from the top. The width of the strip is roughly determined by the amount of FS in the fractions to be applied, in the proportion of 5 mm. to 1 mg. The standard compounds are usually applied in 20-microgram amounts to separate strips 5 mm. wide. The strips are wet with the appropriate solvent mixture and blotted. The material to be chromatographed is concentrated to dryness in a 12-cc. heavy-walled centrifuge tube. A few drops of methanol or methanol-chloroform 1:1 are added and the solution applied to the starting line with a micro-pipette. The urinary residue is transferred quantitatively with several additional washings of solvent. The excess solvent applied is evaporated off in a stream of air. It is desirable to confine the applied material to as small an area as possible. Suitable standard compounds are always run with the residues.

The strips are suspended over the glass plates in the troughs containing one of three solvent systems: (a) benzene saturated with formamide, (b) cyclohexane saturated with formamide, or (c) toluene saturated with propylene glycol. The tanks are tightly

sealed and the strips are allowed to develop for the appropriate length of time. The overflow or effluent is caught in a beaker (30–50 cc.) previously placed in the tank under the strips. This effluent is in turn concentrated in a centrifuge tube, dried in a warm water bath under a stream of nitrogen, reapplied to a strip and developed either in the same system for a shorter period of time or in a more slowly developing solvent system. This procedure is continued for all the effluents of strips until the urinary metabolites present in each of three major fractions have been completely separated. In detail each of the three silica gel fractions is chromatographed as follows:

1. Most polar fraction (A): Prior to application, the acetone-methanol fraction is subjected to a benzene-water partition (11) to remove a portion of interfering (polar) urinary pigments. The benzene fraction contains negligible amounts of FS. The chloroform extract of the water fraction is concentrated and applied to paper strips in the proportion of 1 mg. of FS per 5 mm. of strip width. In the case of the sample in Table 1

TABLE 2. PAPER STRIP CHROMATOGRAPHY OF MORE POLAR AND MOST POLAR METABOLITES (SILICA GEL FRACTIONS A & B)

Chromatogram	Whatman paper #	Solvent system	Solvent for wetting strip	Running time (hrs.)	Standard compounds	Running rate (cm./hr.)
1.	42	Benzene-formamide	Methanol-formamide 1:1	72	Pregnanetriol-3α,17α,21-dione-11,20	0.35
2. Effluent of #1	1 or 540	Propylene glycol-toluene	Methanol-propylene glycol 1:1	48	17-OH corticosterone 11-Dehydro-17-OH-corticosterone	0.23 0.60
3. Effluent of #2	1 or 540	Propylene glycol-toluene	Methanol-propylene glycol 1:1	24	Pregnanetriol-11β,17α,21-dione-3,20 Pregnanediol-17α,21-trione,3,11,20	0.54 1.00
4. Effluent of #3	1 or 540	Benzene-formamide	Formamide	24	Corticosterone	0.39
5. Effluent of #4	1 or 540	Benzene-formamide	Formamide	8	11-Dehydrocorticosterone	0.81

a strip width of 11 mm. would therefore be used. The strip is then wet with methanol-formamide 1:1, and the urinary residue (A) is applied to the starting line and developed through the various steps outlined in Table 2. In each of the subsequent chromatograms, the strip width is usually reduced by 2 or 3 mm. In the instance of the typical sample, the effluent from the 72-hour chromatogram would be applied to a strip 9 mm. wide, and the effluent from the 48-hour chromatogram to a strip 7 mm. wide. However, the strip is rarely reduced to less than 5 mm.

2. More polar fraction (B): This fraction is relatively free of pigment and is applied directly to the paper strips. The chromatographic procedure is the same as that outlined in Table 2 for fraction A. However, we have found it somewhat advantageous to develop the first chromatogram in the toluene-propylene glycol system for forty-eight hours. The material more polar than 17-hydroxycorticosterone is then eluted, applied to paper #42 and developed with benzene-formamide for seventy-two hours. The strip width, as in the case of fraction A, is gradually reduced in size with each chromatogram.

3. Less polar fraction (C): The material of this fraction is applied in the proportion of 1 mg. of FS per 5 mm. of strip width and chromatographed as outlined in Table 3. The

complete elution of whole strips followed by chromatography of the residues therefrom in another solvent system is a useful method for circumventing pigments. It should be pointed out that occasionally the less polar fraction contains, as in the case of the sample presented, large amounts of FS-producing material in the range of 10–20 mg. In our experience, however, there is usually no correlation between the high FS and the amount of urinary metabolites detectable; therefore, the average strip width of 2–3 cm. for the less polar fraction is used.

TABLE 3. PAPER STRIP CHROMATOGRAPHY OF LESS POLAR METABOLITES (SILICA GEL FRACTION C)

Chromatogram	Whatman paper #	Solvent system	Solvent for wetting strip	Running time (hrs.)	Standard compounds	Running rate (cm./hr.)
1.	1 or 540	Propylene glycol-toluene	Methanol-propylene glycol 1:1	16	Pregnanetriol-3α,17α,21-dione-3,20 Pregnanediol-17α,21-tione-3,11,20	0.54 1.00
2. Effluent of #1	1 or 540	Benzene-formamide	Formamide	24	Corticosterone	0.39
3. Effluent of #2	1 or 540	Benzene-formamide	Formamide	16	11-Dehydrocorticosterone Δ^4-Pregnenol-17α-trione-3,11,20 21-OH-pregnenolone	0.81 0.96 1.44
4. Effluent of #3	1 or 540	Benzene-formamide	Formamide	5.3	Pregnanedione-3,20	1.83
4a. Strip #4 eluted and re-run	1 or 540	Cyclohexane-formamide	Methanol-formamide 1:1	16	11-Desoxycorticosterone Allopregnanol-21-dione-3,20	0.40 0.75
5. Effluent of #4	1 or 540	Benzene-formamide	Formamide	2		
5a. Strip #5 eluted and re-run	1 or 540	Cyclohexane-formamide	Formamide	20	Δ^4-Androstenedione-3,17 Androstanedione-3,17 Etiocholanol-3α-one-17	0.31 0.52 0.59

Location of the urinary metabolites: After thorough drying in air at room temperature, the strips are examined under ultraviolet (Mineralight) for areas of ultraviolet absorption. Longitudinal segments of strips are then cut off and tested with any of the following color reagents:

1. Blue tetrazolium chloride (BTZ) for α-ketols (12)
2. Dinitrophenylhydrazine (DNPH) (13)
   a. For α,β-unsaturated ketones (orange color)
   b. Saturated ring ketones (yellow color)
3. Metadinitrobenzene (Zimmermann reagent) for ketones (13) (purple and pink color)
4. Occasionally potassium permanganate or iodine reagent (4) is also employed.

Further characterization of urinary metabolites: After the reactive spots corresponding to the urinary metabolites and standard compounds have been marked and recorded, the unused areas parallel to reacting metabolites are cut out, cut into small pieces and put into a 10-cc. Erlenmeyer flask to which 3 cc. of a methanol-chloroform 1:1 solution is added. After standing two hours, the solvent is decanted through a 2-cc. sintered glass

funnel into a 12-cc. centrifuge tube. Three additional solvent washings of 2.5 cc. are made at fifteen-minute intervals. The eluated (10.5 cc.) are evaporated, and absorption maxima determined in methanol in the ultraviolet from 220 mμ to 330 mμ with the Beckman spectrophotometer (6).

Usually each metabolite is rechromatographed alongside the appropriate standard compound in the appropriate solvent system, for purposes of purification in the determination of adsorption spectra in a solution of H_2SO_4 (14), and for a check of its running rate. In the case of metabolites present in highly pigmented areas, a third chromatogram may be necessary for adequate purification. Each of these metabolites and standard compounds is applied to subdivisions or limbs of paper strips. The width of the individual limbs is determined by the concentration of the metabolites on the initial chromatogram.

After relocation of the standards and urinary metabolites, the latter are eluted and absorption maxima in methanol again determined. An appropriate aliquot of the methanol solution of the metabolite is removed, dried, and the adsorption spectra in H_2SO_4 determined. With the less polar metabolites, the determination of absorption maxima from 380 mμ to 530 mμ, using the Zimmermann reagent, has been helpful in identifying 17-ketosteroids and the presence of other ketone groups. 17-Ketosteroids have a maximum between 500 and 520 mμ, whereas other ketone groups may be indicated by a maximum at 410–420 mμ or 450–460 mμ.

When the identity of an unknown is in question, in addition to a comparison of sulfuric acid spectra, mixed chromatograms with known compounds are run. Only in the case of 11-dehydro-17-hydroxycorticosterone and 17-hydroxycorticosterone have acetates been made and running rates compared. Occasionally a sufficiency of material with suitable solubility has made determination of infrared spectra possible.

RESULTS

Urine collections have been made from a series of normal, healthy subjects of both sexes ranging in age from 20 to 82 years. The formaldehydogenic steroids (FS) per twenty-four hours varied from 15 mg. in the young individuals to 3 mg. in the elderly subjects. Two 24-hour samples from each individual were combined and fractionated by the described method. With fairly constant repetition, certain urinary substances appeared, regardless of the age or sex of the individual. In Tables 4, 5 and 6 the substances which are frequently or always present in the most polar (A), more polar (B) and less polar (C) fractions are outlined in the order of increasing running rates.

The frequency distribution of the metabolites in the A fraction is based on chromatograms of 8 such fractions; that for the B fraction on 17, and for the C fraction on 12. In the A fraction, the number of metabolites present on an individual sample basis ranged from 6 to 9; in the B fraction, from 10 to 14; and in the C fraction, from 12 to 16. Instead of calculating R_f values, the running rates of the metabolites are compared with those of a series of standard compounds.

The absorption maxima in methanol, Zimmermann reagent or sulfuric acid for some of these metabolites are listed in Table 7. The various

maxima for each of the unknowns represent a composite of determinations from individual samples and are not necessarily definitive.

Identification of etiocholanol-3α-one-17 was made by comparison with the infrared spectrum of the pure compound. The four metabolites desig-

TABLE 4. MOST POLAR COMPOUNDS (SILICA GEL FRACTION A), IN ORDER OF INCREASING RUNNING RATES

Standard compounds*	No.	Urinary metabolites	Frequency %
	31	α-Ketol	100
	30	α-Ketol	100
	29	α-Ketol	100
Pregnanetriol-3α,17α,21-dione-11,20	35	α,β-Unsaturated ketone (glycol?)	88
17-OH corticosterone	34	α-Ketol, saturated	75
	25	α,β-Unsaturated ketone (glycol?)	100
11-Dehydro-17-OH-corticosterone			
Pregnanediol-17α,21-trione-3,11,20			
	33	α-Ketol	75
Corticosterone	32	α-Ketol	88

* In Tables 4, 5 and 6 the location of the compounds run as standards indicates their running rates relative to the numbered urinary metabolites.

TABLE 5. MORE POLAR COMPOUNDS (SILICA GEL FRACTON B), IN ORDER OF INCREASING RUNNING RATES

Standard compounds	No.	Urinary metabolites	Frequency %
	31	α-Ketol	53
	30	α-Ketol	83
	29	Pregnanetetrol-3,11,17,21-one-20	100
Pregnanetriol-3α,17α,21-dione-11,20	28	Pregnanetriol-3,17,21-dione-11,20	100
	27	α-Ketol	65
17-OH corticosterone	26	17-OH corticosterone	100
	25	α,β-Unsaturated ketone (glycol?)	77
11-Dehydro-17-OH-corticosterone	24	11-Dehydro-17-OH-corticosterone	100
Pregnanetriol-11α,17α,21-dione-3,20	23	α-Ketol	65
Allopregnanetriol-3β,17α,21-one-20			
	22	α-Ketol	94
Pregnanediol-17α,21-trione-3,11,20	21	α-Ketol	77
	20	α-Ketol	71
Corticosterone	19	α-Ketol, α,β-unsaturated ketone	94
	18	α-Ketol	82
	17	α-Ketol, α,β-unsaturated ketone	53

TABLE 6. LESS POLAR COMPOUNDS (SILICA GEL FRACTION C), IN ORDER OF INCREASING RUNNING RATES

Standard compounds	No.	Urinary metabolites	Frequency %
Pregnanetriol-11β,17α,21-dione-3,20	16	α-Ketol	58
	15	α,β-Unsaturated ketone	100
	14	α-Ketol	75
	13	α,β-Unsaturated ketone	75
Pregnanediol-17α,21-trione-3,11,20	12	α-Ketol	75
	11	17-Ketosteroid, unsaturated	100
Corticosterone			
	10	α-Ketol?	100
11-Dehydrocorticosterone			
	9	α-Ketol?	92
Δ^4-Pregnenol-17α-trione-3,11,20	8	α-Ketol?	100
21-OH-pregnenolone	7	α-Ketol?	92
Desoxycorticosterone			
	6	α-Ketol, α,β-unsaturated ketone	100
Allopregnanol-21-dione,3,20			
	5	α-Ketol, unsaturated	92
Δ^4-Androstenedione-3,17	4	C 21-CH_3?	83
	3	C 21-CH_3?	50
Androstanedione-3,17			
3α-OH-etiocholanol-3α-one-17	2	3α-OH-etiocholanol-3α-one-17	100
	1	17-Ketosteroid	83

nated as known compounds (Table 5) were identified by a comparison of color reactions, running rates, and sulfuric acid spectra. Metabolite #29, a saturated substance with a positive α-ketol reaction, had sulfuric acid maxima of 330 mμ, 415 mμ and 510 mμ, identical with those of pregnanetetrol-3α,11β,17α,21-one-20, which has previously been isolated by Lieberman *et al.* (15) from urine after the administration of adrenocorticotropic hormone. Its infrared spectrum was also similar to the spectrum of that compound. Metabolite #28 had a running rate and maxima (330 mμ, 415 mμ) identical with those of pregnanetriol-3α,17α,21-dione-11,20, which has also been described by Lieberman (15) and Schneider (16, 17). Its infared spectrum was also identical with that of the aforementioned compound. 17-Hydroxycorticosterone and 11-dehydro-17-hydroxycorticosterone have been reported frequently as being present in urine (5,15,16,18,19). Metabolite #21 is most likely pregnanediol-17α,21-trione-3,11,20, since the running rates and sulfuric acid maxima of these compounds are very similar. Pregnanediol-17α,21-trione-3,11,21 has already been identified in urine extracts by Schneider (17). Metabolite #19 is interesting, in that it possesses an α,β-unsaturated ketone, has sulfuric acid maxima and a

TABLE 7. MAXIMA IN METHANOL, H_2SO_4 AND ZIMMERMANN REAGENT

No.	MeOH	H_2SO_4	No.	MeOH	H_2SO_4
35	240	240, 280–290, 390–400	18		265, 310
34		265, 310	17	?	285–290
			16		(265), 340, 400–405, 505–510
33	?	280–285, (385–400)*	15	242, 260, 295	265, 320, 405
32		260, 310–320	14		
31		260–270, 330–335, 385–395	13		275, 345
30		260–265, 310–315, (390–400)	12		
29		330, 415, 510†	11	247	255, (390–405)
28		330, 415†	10		
27		265–270, 305, 425	9		310, 365, 430
26	240	280–285, 395, 470–475†	8		
25	238–240	285, 385–395, 440	7		
24	240	280–285, 345, 410–415†	6	238–240	285, 365–375
23		310–330, 420	5		
22		310, 435	4		
21		265, 315–335, 415, (495–500)	3		
20		295	2		Max. 510 Zim.‡
19	240	280–285, 335–340, 365–390, 445–450	1		Max. 510 Zim.‡

* Parentheses indicate plateau rather than maximum.
† Maxima similar to those reported by Zaffaroni (14).
‡ Maximum in Zimmermann reagent.

running rate closely resembling those of corticosterone. Caffeine, identified by infrared, is usually eluted from the silica gel column with acetone. On paper its running rate is faster than that of corticosterone. It reacts very weakly with BTZ and gives a yellow reaction with DNPH. It has an absorption maximum in methanol at 270 mμ. In the less polar fraction a substance with a running rate similar to Δ^4-androstenedione regularly appears and reacts with alkali to produce a pink-orange color. There is also some question as to the steroidal nature of metabolite #15.

There is an obvious overlapping of the running rates of some metabolites. When unknowns in separate fractions might possibly be identical the same number has been assigned to each. By the same token, supposedly structurally different compounds with similar running rates have been numbered individually. Thus the three very polar α-ketols in fraction A are assigned the numbers given to the three very polar α-ketols in fraction B since, to date, the running rates, color reactions and sulfuric acid spectra have been similar. Metabolite #25 is also present in both of these fractions.

In the case of #34 and #35, however, these metabolites are not 17-hydroxycorticosterone and pregnanetriol-3α,17α-21-dione-11,20, respectively. Whether metabolites #33 and #32 in fraction A are similar or not to #21 and #20 in fraction B, has not been determined. Those metabolites in fraction C with running rates similar to those in fraction B appear to be structurally different.

Attempts at quantitative measurement consisted of rough visual estimation of the relative amounts of color produced with the various reagents by the different metabolites. Generally, in fraction A unknowns #34 and #35 are most conspicuously present. In the C fraction, unknowns #15 and #11 occur in the largest quantity, with unknown #6 present in a somewhat smaller amount. In the case of the B fraction, pregnanetriol-3α,17α,21-dione-11,20 and pregnanetetrol-3α,11β,17α,21-one-20, are the most abundantly occurring urinary metabolites. Usually no other marked quantitative differences are noted. Like Schneider (19), we have found that 11-dehydro-17-hydroxycorticosterone appears to have the quantitative edge over 17-hydroxycorticosterone.

DISCUSSION

The use of glucuronidase hydrolysis has made possible the determination of corticosteroid patterns of extracts from two-day collections of human urine, whereas previously a prolonged collection would have been required.

Preliminary silica gel fractionation has been very useful in eliminating some of the difficulty with urinary pigments and distributing, on the basis of polarity, numerous metabolites. This has provided a convenient preliminary separation of some metabolites which are structurally different, but which have similar running rates on paper. Although we prefer to continue the use of the more complicated chromatographic system outlined, it obviously may be simplified.

The application of Zaffaroni's paper chromatographic technique to the separation of urinary corticosteroids has been characterized by the relative simplicity and flexibility of the methods. Certain variations have been made which seem to be useful when dealing with urinary extracts. In the case of the most polar metabolites the benzene:formamide system, used with paper #42 and developed for seventy-two hours, has provided a separation of some of the metabolites (*i.e.*, tetrahydrocortisone and 17-hydroxycorticosterone) which do not separate well with the much faster developing chloroform-formamide system. If less developing time with the benzene:formamide system is desired, a good separation of the very polar material may be had in thirty hours by increasing the tank temperature. However, we have used chloroform:formamide to separate further some metabolites slower than pregnanetetrol-3α,11β,17α,21-one-20.

Obviously a choice of useful developing solvent combinations is available, depending upon the speed desired and nature of the material to be separated. We prefer solvent systems which may be slower but give better resolution of metabolites. In Tables 2 and 3, for example, it may be noted that the running rates of standard compounds in the various solvent systems never exceed 1.83 cm./hour.

The development of paper chromatograms in the order of the most polar to the less polar has been convenient, although the procedure might well be reversed. In the case of the most-polar A fraction, such a reversal may be useful in eliminating difficulty occasionally encountered when profuse amounts of caffeine are present.

Obviously the color reagents are most sensitive for steroids which possess α-ketol and unsaturated groupings. The Zimmermann reagent will detect 3-ketone and 20-ketone-containing steroids, if they are present in 50-μg. amounts per 5-mm. strip width in the case of a mono-ketone, and 30–40 μg. per 5-mm. strip width in the case of a di-ketone. Saturated glycerols will react with potassium permanganate reagent, but a steroid of the nature of pregnanediol-3,20 cannot be located by the methods here presented. Unreacting areas have therefore been cut from strips, eluted, and sulfuric chromogen spectra determined. With one exception, this technique has produced negative results. This exception occurred in the 24-hour paper chromatogram of the B fraction from which a metabolite with a sulfuric acid spectrum closely resembling that of pregnanetriol-3α,17α,20α has been recovered several times. Of course the possibility exists that an undetectable steroid may have the same running rate as a reactive one. This is less likely to occur with the slower running metabolites (fractions A and B), which separate well in our system.

Although the amounts of metabolites present in a given 48-hour collection are in sufficient quantity to determine patterns, often there are insufficient amounts for more clear-cut characterization, particularly when pigment has to be removed. Therefore, it is hoped that with pools of eluted metabolites, further characterization of some of the metabolites can be made. This procedure should be helpful in finally deciding the difference or similarity of metabolites with similar running rates, which have not definitely been characterized as different. Some of the sulfuric acid maxima, as previously pointed out, are only tentative, being the maxima obtainable with the amount of a given metabolite present in a 48-hour urine collection.

Nevertheless, the repetition of the metabolic pattern, regardless of age or sex, obtained by the methods described has been relatively consistent. Thus 30 of the 35 compounds characterized appear in 70 per cent or more of the samples analyzed and 11 have been seen invariably. None has appeared less than 50 per cent of the time. The occasional non-ap-

pearance of certain substances may be due to inadequate quantities for positive identification. Occasionally additional metabolites have been present in some subjects. Therefore, it is felt that the method may very well lend itself to determination of urinary steroid patterns in studies of various disease states or studies of effects of hormone administration. In a subsequent paper, results of this type of study will be presented. Meanwhile, work is in progress to identify further some of the many detectable metabolites regularly occurring in normal individuals.

The rather large array of substances indicated in this analytical system is somewhat in contrast to the findings of others (15, 17, 18) published to date. It is possible that previous work has been successful in characterizing only major urinary corticosteroids such as cortisone, hydrocortisone and particularly their tetrahydro derivatives, because of the use of analytical systems in which less prominent constituents are masked. Of course we recognize that full identification of the newly characterized substances is essential before certain establishment of the large array presented. Some of the compounds herein called steroid metabolites may indeed be non-steroidal, but on the basis of characteristic color reactions the unknown compounds behave like known steroids.

SUMMARY

A method has been devised for routine determination of steroid patterns in a 48-hour collection of urine. This includes: glucuronidase hydrolysis, continuous extraction of the urine at pH 1.0 with methylene chloride, a preliminary fractionation on a silica gel column and further separation of metabolites in these fractions by means of paper strip chromatography. An analysis of a series of urines has demonstrated the reproducibility of the metabolic patterns in normal individuals of both sexes ranging in age from 20 to 82 years.

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PLASMA NEUTRAL 17-KETOSTEROIDS

I. TECHNIQUE OF ESTIMATION

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ALTHOUGH there have been rapid advances in the determination of blood 17-hydroxycorticosteroids (1, 2, 3), 21-hydroxy-20 ketosteroids (2) and 3-ketosteroids (4, 5), little is known about the concentration of 17-ketosteroids in blood. West and colleagues reported no measurable amounts of 17-ketosteroids in peripheral human blood (6). Testosterone and Δ^4-androstenedione have been identified in spermatic vein blood from dogs (7). Dumazert and Valensi have published values of 800 μg. to 1,200 μg. per 100 ml. for neutral 17-ketosteroids in human peripheral plasma, but found no correlation with urinary 17-ketosteroid excretion (8).

This communication deals with the development of a method for the quantitative determination of 17-ketosteroids in peripheral plasma. The steroids are liberated by direct acid hydrolysis of plasma and the extracts are purified by column chromatography. A micro-modification of the Zimmermann reaction is used. Overestimation is avoided by Allen's spectrophotometric technique (9).

MATERIALS AND METHODS

Reagents

1. Ethyl ether, U.S.P., redistilled from 2,4-dinitrophenylhydrazine and washed with 1 per cent aqueous ferrous sulfate.
2. Chloroform, reagent grade, redistilled from 2,4-dinitrophenylhydrazine.
3. Ethanol, absolute, redistilled from potassium carbonate (K_2CO_3) and kept in a refrigerator.
4. Hexane (Eastman Kodak Co.), redistilled.
5. Magnesia-silica gel adsorbent ($MgO \cdot SiO_2$), "Florisil," 60/100 mesh (Floridin Company, Warren, Pa.). This adsorbent has been activated at 650° C. for 105 minutes by the manufacturer. It should be kept in tightly stoppered bottles and washed with chloroform prior to use, as described subsequently.
6. Meta-dinitrobenzene (Eastman Kodak Co.). Recrystallize twice, as follows: Dissolve 20 grams in 750 ml. of 95 per cent ethanol. Heat to 40° C. and add 100 ml. of approximately 2 N sodium hydroxide. After five minutes the solution is cooled. Add 2,500 ml. of distilled water. Collect the precipitate on a Buchner funnel and wash several times with distilled water. Dry on filter paper in a desiccator. Dissolve in a few milliliters of absolute ethanol and reprecipitate in the cold. Wash again with water on a Buchner funnel and dry in a desiccator. A 1 per cent solution in absolute ethanol is made fresh each day. Keep in a dark bottle.

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7. Potassium hydroxide pellets, C.P. (2 grams added to 10 ml. of absolute ethanol). Shake five minutes in glass-stoppered tube and centrifuge. Make fresh each day just before use.
8. Dehydroepiandrosterone acetate (Schering Corp.)—50 mg. per cent solution in absolute ethanol for use as a standard.

Special equipment

1. *Lowry-Bessey diaphragm attachment for Beckman DU spectrophotometer.* The design and operation of this device have been published elsewhere (10). (Pyrocell Mfg. Co., 207 E. 84th St., New York, N. Y.)
2. *Special cuvettes for Beckman DU spectrophotometer:* Inside measurements 40×4.3 ×10 mm. (light path 10 mm.). Silica cuvettes were used in the present study, but this is probably not necessary (Pyrocell Mfg. Co., New York, or A. H. Thomas Co., Philadelphia, Pa.). These cuvettes are used in the standard Beckman cuvette carrier. Their vertical position in the carrier must be calibrated, to make sure the light beam passes through the center of the 0.5 ml. of solution. Shorter cuvettes (25 mm.) made by Pyrocell may also be used. These require a special cuvette carrier but are easier to center.
3. *Nelson-Samuels flasks:* Pyrex 50-ml. Erlenmeyer flasks with a center-well blown in the bottom. A well 33 mm. long×6 mm. in diameter is optimal for the final volume of 0.5 ml. (Macalaster-Bicknell Co., Syracuse, N. Y.).
4. *Chromatography tube:* An 18-inch length of Pyrex tubing, 10 mm. inside diameter, with one end constricted so as to hold a plug of glass wool.

Procedure

In normal men and women, a 6 to 10 ml. sample of oxalated or heparinized plasma is used. In children with untreated congenital adrenal hyperplasia, 1 to 5 ml. of plasma has been satisfactory. The method does not appear to be sufficiently sensitive to measure the levels in preadolescent children.

The plasma is pipetted into a 250-ml. Erlenmeyer flask, to which is added 25 ml. of 10 per cent aqueous hydrochloric acid and two glass beads. With volumes of plasma over 10 ml. the amounts of 10 per cent hydrochloric acid are proportionately increased. Boil the mixture on a hot-plate for twelve minutes, swirling the flask occasionally to prevent bumping. Cool the flask by running tap water around its base and wash the contents into a 250-ml. centrifuge bottle. Add 25 ml. of ether and stir with a stirring rod. Centrifuge briefly to break the emulsion. Repeat this three more times, ending with 100 ml. of ether extract. Using a separatory funnel, wash with three 25-ml. portions of 10 per cent sodium hydroxide solution. Discard the sodium hydroxide wash. Wash with three 50-ml. portions of distilled water, or until the wash water is neutral to litmus. Draw off all ether into a 250-ml. Erlenmeyer flask. Rinse the funnel with a few drops of ether. Add two glass beads and evaporate to dryness on a steam bath. Take up the residue in 20 ml. of 70 per cent ethanol. Wash three times with 15 ml. of hexane, using a small separatory funnel. Discard the hexane. Add 10 ml. of distilled water to the 70 per cent ethanol in the separatory funnel. Extract the ethanol with three 15-ml. portions of chloroform. Discard the ethanol layer and reduce the chloroform to about 12 ml. volume in an Erlenmeyer flask on the steam bath. Prepare a chromatography column consisting of 3 grams of Florisil in a Pyrex tube of 10 mm. inside diameter. Wash the Florisil with 30 ml. of chloroform, then add the sample in chloroform. When the upper meniscus of this solution has nearly reached the Florisil, add 35 ml. of chloroform. Collect the approx. 47 ml. in a flask. This eluate is evaporated on a steam bath in a special Nelson-Samuels flask

TABLE 1. PROCEDURE BY WHICH PLASMA EXTRACTS, REAGENT BLANK STANDARDS AND STANDARD BLANK ARE CARRIED THROUGH THE REACTION WITH THE ZIMMERMANN REAGENTS

(See section on "Reagents" for preparation of standard (dehydroepiandrosterone acetate) and MDB (metadinitrobenzene).)

	Abs. EtOH (ml.)	Alc. KOH (ml.)	MDB (ml.)	Standard (ml.)	Incubated 1 hr. at 30° C.	Add abs. EtOH (ml.)
Plasma extracts and reagent blank	.02	.02	.02	—		.44
Blank for standards	.2	.2	.2	—		4.4
Standard—2.0 μg.	.16	.2	.2	.04		4.4
Standard—8.0 μg.	.04	.2	.2	.16		4.4

with a center-well blown in the bottom. When the chloroform has evaporated, wash down with a few more ml. of chloroform so that all of the sample is in the center well, and evaporate to dryness on a steam bath. Place flask upright in a vacuum desiccator for two to four hours.

The Zimmermann procedure is as follows: Reagents are added as shown in Table 1 to the center well of the special flasks and to the standards. The color reaction of the standards may conveniently be carried out in glass-stoppered 10-ml. graduated cylinders. The center wells of the Nelson-Samuels flasks are thumped vigorously to dissolve the samples, after which the flasks are stoppered tightly and with the standards are placed in a water bath at 30° C. for sixty minutes. At the end of this time the samples are diluted with absolute ethanol as noted in Table 1. Again the center wells are thumped vigorously and the cylinders containing the standards are inverted. Samples are transferred to cuvettes by mouth suction with a fine-tipped glass tube attached to a rubber tube. Optical density measurements are then made *as rapidly as possible* in the Beckman DU spectrophotometer. Measurements are made on each standard and sample at 460 mμ, 520 mμ and 580 mμ. The standards are read against a blank prepared from absolute ethanol, and the samples are read against a blank prepared by subjecting a sample of distilled water to the entire extraction procedure (see Table 1). As shown in Figure 1, there is good linearity of the dehydroepiandrosterone acetate standard between 1 μg. and 5 μg. Derived optical density is calculated by the formula of Allen (9): Derived $OD = OD_{520} - \left(\frac{OD_{460} + OD_{580}}{2}\right)$. The calibration curve is prepared by plotting derived OD values for standard on the ordinate and μg. values of standard on the abscissa. Derived OD values for samples are read off this calibration curve and the result in μg. per 100 ml. of plasma calculated.

RESULTS

Preliminary experiments confirmed the observation of Nelson, Samuels and Reich that certain C-19 steroids could be separated from compound F

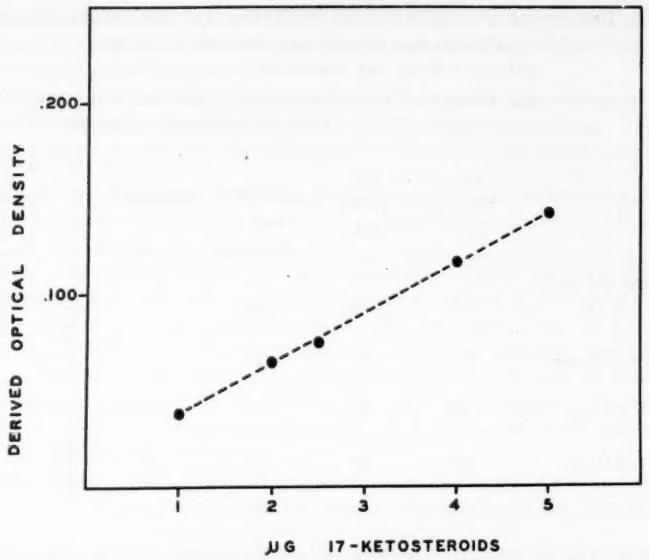

FIG. 1. Calibration curve showing dehydroepiandrosterone acetate in μg. plotted against derived optical density. See "Method" section for explanation of the latter term.

TABLE 2

(The fractions were prepared on a column of 10 mm. inside diameter, using 3 Gm. of Florisil. The adsorbent was thoroughly washed with chloroform. The pure steroid was then added in 12 ml. of chloroform, after which the solvent aliquots were put on the column.)

Fraction No.	Solvent fractions (12 ml. each)	Compound F (100 μg.)	Androsterone (100 μg.)	Dehydroepiandrosterone acetate (100 μg.)	Cholesterol (100 μg.)
1	$CHCl_3$	0	++	+	+++
2	$CHCl_3$	0	+++	++++	+++
3	$CHCl_3$	0	++	++	+
4	$CHCl_3$	0	+	0	0
5	2% Ethanol in $CHCl_3$	+++	0	0	0
6	2% Ethanol in $CHCl_3$	+++	0	0	0
7	10% Ethanol in $CHCl_3$	+++	0	0	0
Color reaction used		Porter-Silber	Conc. H_2SO_4 at 100° C.	Dirscherl-Zilliken	Liebermann-Burchard

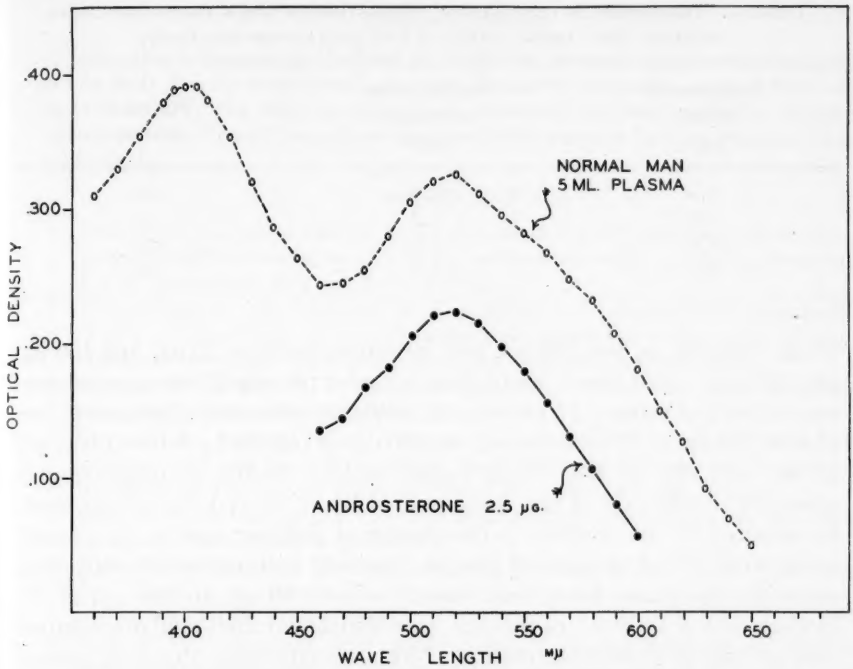

FIG. 2. Spectrophotometric curves of Zimmermann reaction, with 2.5 μg. of androsterone and with 5 ml. of plasma carried through the entire procedure.

by chromatography with a chloroform-ethanol system (11). Table 2 shows the results obtained when pure samples of compound F, androsterone and dehydroepiandrosterone acetate were chromatographed on Florisil with a chloroform-ethanol system. Accordingly, the present technique utilizes the chloroform fraction exclusively in the separation of plasma 17-ketosteroids.

Figure 2 shows the spectral absorption characteristics of the Zimmermann reaction when carried out on crystalline androsterone and on adult male plasma subjected to the extraction procedure herein described. The crystalline androsterone was found to absorb maximally at 515 mμ, and the extract of adult male plasma at 520 mμ (12). Spectral curves on extracts of plasma from newborn infants show maxima at 500 mμ. The latter finding suggests that qualitative differences exist between the Zimmermann-positive substances in the plasma of adult males and those in newborn infants.

Table 3 shows recoveries of pure steroid when carried through the procedure.

Normal values for peripheral adult plasma in men have varied between

TABLE 3. RECOVERIES OF CRYSTALLINE ANDROSTERONE WHEN ADDED TO PLASMA SAMPLES AND CARRIED THROUGH THE EXTRACTION PROCEDURE

Androsterone added (μg.)	Androsterone recovered (μg.)	Per cent recovery
2.5	2.6	104
2.0	1.7	85
3.0	2.8	93
3.5	3.3	94

40 μg. and 130 μg. per 100 ml. and in women between 25 μg. and 100 μg. per 100 ml. A consistent parallelism between plasma 17-ketosteroid concentration and urinary 17-ketosteroid excretion was noted. Concentrations of over 100 μg. of 17-ketosteroids per 100 ml. of plasma have been observed in the first day of life. These values rapidly decline to unmeasurable quantities at the end of the first week of life. No 17-ketosteroids could be detected by this method in the plasma of children aged $3\frac{1}{2}$ to 5 years, using 20 to 22 ml. samples of plasma. Patients with untreated congenital adrenal hyperplasia have been found to have 80 μg. to 360 μg. of 17-ketosteroids per 100 ml. of plasma. An adrenalectomized-oöphorectomized woman had no detectable plasma 17-ketosteroids when 19 ml. of plasma were extracted.

The parallelism of plasma concentrations with urinary 17-ketosteroid findings in normal children and adults, in patients with congenital adrenal hyperplasia, and in the adrenalectomized-oöphorectomized subject suggests that the Zimmermann-positive steroids of plasma as herein described bear a very close relationship to urinary 17-ketosteroids. Detailed data on the plasma 17-ketosteroid level in children and adults, and its relationship to urinary 17-ketosteroid excretion will be presented in another paper (13).

SUMMARY

A method is described for the measurement of neutral 17-ketosteroids in peripheral plasma. Between 6 and 10 ml. of plasma from a normal adult suffice for the determination. Values for normal men have been found to be 40–130 μg. per 100 ml. and for normal women, 25–100 μg. per 100 ml.

Addendum

Migeon has recently isolated from acid-hydrolyzed plasma, by paper chromatography, a compound with the same Rf as dehydroepiandrosterone (between 15 and 20 μg. per 100 ml of plasma) (14).

Acknowledgments

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METABOLISM OF TESTOSTERONE BY NEOPLASTIC HUMAN PROSTATE*

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ONE of the most important contributions to the treatment of inoperable human carcinoma has been the application of endocrinologic control measures to prostatic carcinoma, developed by Dr. Charles Huggins twelve years ago. Orchiectomy and/or estrogen therapy appear to have increased the survival rate in this type of metastatic carcinoma (1, 2). Further benefit to some of these patients has been obtained more recently by total adrenalectomy, thereby insuring the removal of all known endogenous sources of androgenic hormones (3). It is believed that the favorable response manifested by some prostatic carcinomas to antiandrogenic therapy depends upon the degree to which the malignant tissue retains dependence upon the specific hormonal stimuli characteristic of the tissue of origin. The improvement of some premenopausal patients with metastatic breast cancer after oophorectomy lends additional support to this concept of residual sensitivity to hormones in cancers arising in accessory sex organs.

These clinical observations are of utmost importance for they indicate a partial lack of autonomy in carcinomas. The desirability of gaining information concerning the mechanism of action of steroids upon individual target organs and malignant tumors appears obvious. Not only might it be possible to select more wisely for therapy those patients with tumors susceptible to endocrinologic control, but more effective use of sex hormones or their analogues in tumor chemotherapy might be achieved.

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† The authors gratefully acknowledge the receipt of authentic samples of steroids from U. S. Vitamin Corp. and from Organon, Inc.

EXPERIMENTAL

With these objectives in mind we have inquired into the nature of the interaction between surviving specimens of human tissues and testosterone *in vitro*. We have chosen prostate (which is known to be a male sex hormone-dependent tissue) for the initial studies, using thin sections sliced from surgical specimens in a Stadie-Riggs microtome.

The slices are incubated aerobically in a Warburg bath at 37.5° C. for three hours, in 15 to 20 ml. of Krebs-Ringer-phosphate medium buffered at pH 7.3, which contains 20,000 units of penicillin. Free testosterone is added just prior to incubation in 5–20 mg. amounts dissolved in 0.5 ml. of propylene glycol. Control flasks are run, utilizing tissue aliquots boiled immediately after addition of tissue to substrate. Under these conditions quantitative recovery of added testosterone is possible (Table 1).

TABLE 1. RECOVERY OF TESTOSTERONE FROM HEAT-INACTIVATED TISSUE-SUBSTRATE

Control: (Tissue plus substrate boiled prior to incubation)	Gm. tissue (dry wt.)	Mg. testosterone added	Mg. testosterone recovered	Per cent recovery
Boiled 10 minutes	1.20	20.0	19.0	95
	1.60	16.3	15.0	97
	.28	5.0	4.6	92
	.31	10.3	9.8	95

At the conclusion of an experiment the flasks are boiled ten minutes, then rapidly frozen until analysis. Testosterone is subsequently recovered by means of conventional methods of extraction, followed by paper chromatographic separation and quantitation, using the absorption at 240 mμ of the alpha-beta unsaturated ketone in the Beckman spectrophotometer (4). With identical procedures no testosterone has been recoverable from as much as 100 Gm. of unincubated hypertrophic or malignant prostatic tissue.

In all cases the dry weight of the lyophilized lipid-free tissue has been determined, as well as the desoxypentosenucleic acid (DNA) content, using the diphenylamine reaction applied to hot trichloroacetic acid extracts (5, 6).

The experiments are reported in terms of the milligrams of testosterone which have been metabolized and which no longer are recoverable. Evidence has been obtained that conjugation of testosterone does not occur and cannot account for any disappearance of free steroid.

CLINICAL OBSERVATIONS

Our case material includes a series of tumors from patients with benign hypertrophy and carcinoma of the prostate, and clinical observations extending back for six to thirty-six months on most patients. These are summarized as follows:

A. *Benign prostatic hyperplasia*

No. 1—Bar., 61, had a history of urinary obstruction for two years. The prostate

was moderately and diffusely enlarged. Pyelograms revealed no urinary tract or osseous pathologic conditions. On May 16, 1951 a perineal prostatectomy was performed; on the prostatic specimen obtained, the analyses were carried out. Pathologic report: benign fibromuscular and glandular hyperplasia, chronic prostatitis, with no evidence of malignancy.

No. 2—Doo., 73, was hospitalized because of frequency of urination. The prostate was asymmetrically enlarged, fixed, and very firm. X-ray films showed a normal chest. Pyelograms showed bilateral hydronephrosis and marked compression of the vertebral body of D12 and L1. On January 14, 1952 a suprapubic prostatectomy was performed. There was only evidence of benign hyperplasia in three sections of the gland, from which the tests were made.

No. 3—Dri., 79, was admitted for painless hematuria of two days' duration. The prostate was diffusely enlarged. Cystoscopy showed blood coming from the left ureter, and pyelograms showed a poorly functioning atrophic left kidney. On October 25, 1951 a retropubic prostatectomy was performed. The specimen showed benign hyperplasia and chronic prostatitis. Subsequent x-ray films showed generalized osteoporosis of the dorsal spine with minimal collapse of D11. There was no recurrence of urinary symptoms by July 1952.

No. 4—Ray., 52, had dysuria, frequency and incontinence of six months' duration, and acute retention on admission. An enlarged intravesical prostate was seen by cystoscopy and by x-ray examination. Chest films showed a cystic lesion, possibly a benign giant-cell tumor, in the right tenth rib. On June 19, 1952 a suprapubic resection of the prostate was carried out. Benign hyperplasia of the gland was reported by the pathologist.

No. 5—Tar., 73, was admitted because of acute urinary retention of five days' duration. The prostate was diffusely enlarged, and x-ray examination of the chest, abdomen and urinary tract were not remarkable. A suprapubic prostatectomy was performed July 2, 1952, the gland showing benign hyperplasia and chronic prostatitis.

No. 6—Mil., 80, was admitted for treatment of hematuria of one year's duration. The prostate was markedly enlarged throughout. On April 14, 1952 a transurethral resection was performed, and some of the pieces used for analysis. Benign prostatic hyperplasia was reported by the pathologist.

No. 7—Ree., 59, was admitted for treatment of frequency and nocturia of four years' duration. The prostate was moderately and diffusely enlarged. Chest films showed a small area of translucence in the right first rib, which did not resemble metastatic tumor. A suprapubic prostatectomy was carried out July 19, 1952. The pathologist reported benign prostatic hyperplasia and no evidence of malignancy.

No. 8—Goo., 81, had intermittent hematuria of ten years' duration. The right lobe of the prostate was tender, enlarged, irregular and firm. Cystoscopy revealed a bladder papilloma. X-ray examination showed extensive Paget's disease of the pelvis, skull and femora, without definite evidence of metastases. Two transurethral procedures were carried out, the first to remove a transitional-cell bladder papilloma, and the second for benign hyperplasia of the prostate; there was no evidence of malignancy in the cuttings used for analysis. Orchiectomy was also performed on March 24, 1952. His initial serum acid phosphatase level was slightly elevated (4.4 G.U.), and alkaline phosphatase was 1.83 B.U. The acid phosphatase level fell to 2.8 G.U., and alkaline phosphatase rose to 7.47 B.U. at the time of discharge on April 6.

No. 9—Ell., 73, was admitted because of acute and chronic urinary retention. The prostate was diffusely enlarged to 8–10 cm. in diameter, without nodules or fixation.

Complete skeletal x-ray films showed no metastases, and the serum acid phosphatase level was 3.6 to 1.6 G.U. preoperatively. On July 29, 1952 perineal prostatectomy was performed. Benign hyperplasia and slight chronic prostatitis were reported by the pathologist.

B. Prostatic carcinoma

No. 1—Sey., 38, complained of slowing of urination, first noted in 1934 for a short while, recurring more persistently in 1949. In 1949 and 1950 he had recurrent painful swelling of the left breast, with loss of weight and strength; a fixed, markedly enlarged, asymmetrical prostate was noted at this time by his physician. A transurethral resection revealed carcinoma in November 1951 and he was started on stilbestrol December 1, 1951. The initial dose of 1 mg. daily was increased to 20 mg. daily on December 19, 1951. Pyelograms showed mild hydronephrosis; no bony metastases were identified either in these films or in chest and abdominal films. On January 16, 1952 a radical perineal prostatectomy was carried out, which revealed a poorly differentiated carcinoma, from which our analyses were made.

No. 2—Hur., 78, was admitted because of acute retention of forty-eight hours' duration. The prostate was diffusely enlarged to three times normal size. Lumbar spine x-ray films showed numerous osteoblastic metastatic areas, originally reported as "sclerosis." On July 31, 1951 a suprapubic prostatectomy was carried out, followed by orchiectomy September 11. In one small area of the prostate (size not measured) there was well differentiated adenocarcinoma, with typical benign hypertrophy in the rest. The testes were histologically normal.

No. 3—Dow., 71, was admitted in a semicomatose state, with a history of intermittent treatment for prostatic cancer by means of estrogen pills in unknown amounts by an outside physician. There were extensive osteolytic and osteoblastic metastases throughout the skeleton, and the liver was markedly enlarged. The patient died three days after admission and tissue for analysis was obtained a few hours post mortem from extensive hepatic metastases. The primary source was a 4×6 mm. carcinoma in the prostate. Gynecomastia and testicular atrophy were also present at autopsy.

No. 4—Ber., 58, is to be reported in detail elsewhere (7). In January 1950 he responded well to orchiectomy and estrogen administration for disseminated prostatic carcinoma (proved by transurethral resection). He gained a total of 22 pounds during the next eighteen months, and his serum acid phosphatase level fell from 34.5 to 3.0 G.U. In the autumn of 1951, bone pain recurred, he lost 25 pounds in weight, and the serum acid phosphatase returned to levels of 7–8 G.U. When estrogen therapy was discontinued, the serum acid phosphatase again rose to 30 G.U. A test dose of 25 mg. of testosterone propionate intramuscularly caused marked accentuation of bone pains. On January 16, 1952 tissue was obtained for analysis by transurethral resection, after two days' treatment with 3.0 mg. of ethinyl estradiol daily. On January 25, 1952 total bilateral adrenalectomy was performed, which was followed by complete relief of bone pain for another eight months, and by a persistently normal serum acid phosphatase level. The osseous metastases continued to grow very slowly in this interval. Postoperative urinary 17-ketosteroid excretion, while the patient was receiving 25 mg. of cortisone daily, varied between 1.0 and 2.0 mg. per twenty-four hours. ACTH therapy (total dose, 200 mg.) did not change the 17-ketosteroid excretion.

No. 5—Haw., 52, is also to be reported in detail elsewhere (8). The tissue tested was from a cutaneous metastasis of prostatic adenocarcinoma, which had shown no striking remission after orchiectomy, estrogens, and total adrenalectomy, over a period of two and a half years. Crises of adrenal insufficiency occurred on two occasions, when ACTH

was administered and cortisone substitution discontinued. Postoperative urinary 17-ketosteroid excretion was usually between 3 and 4 mg. per twenty-four hours, during maintenance dosage of 50–75 mg. of cortisone per day.

No. 6—Cha., at the age of 56 in 1937, had a diagnosis of prostatic adenocarcinoma made by competent pathologists at the time of a perineal prostatectomy for urinary obstruction. During the interval, until March 27, 1952, when he was admitted because of a partial hemiplegia, he had no estrogen therapy and his testes were intact. The right lobe of the prostate was firm, fixed and enlarged 1½ times. The hemiplegia soon cleared after admission, and on April 10, 1952 transurethral resection of the prostate was carried out, with bilateral orchiectomy. Adenocarcinoma was present in the portions of the gland used for analysis; the testes were normal. Subsequently, severe hot flashes developed, often occurring several times in an hour, with occasional intervals of freedom lasting a few hours. This complaint brought him back to our out-patient department August 5, 1952.

TISSUE OBSERVATIONS

Every specimen of histologically verified benign prostate hyperplasia which has been tested has metabolized significant amounts of testosterone (Table 2). The disappearance of free steroid has in each case far exceeded the limits of error of the methods used for recovery of testosterone. The

TABLE 2. METABOLISM OF TESTOSTERONE BY BENIGN HYPERPLASTIC PROSTATIC TISSUE

Case No.	Patient		Gm. tissue (dry wt.)	Mg. testosterone				Serum acid phosphatase (g.u.)	Bone roentgenograms	Endocrine therapy
	Name	Age		Added	Metabolized	Metab./Gm./hr.	Metab./mg. DNA/hr.			
1	Bar.	61	1.07	9.3	5.84	1.82	.051	—	—	None
2	Doo.	73	2.70	20.0	5.96	0.71	.061	3.7	Compression D12, L1	None
3	Dri.	79	.52	5.0	3.99	2.55	.069	2.4	General osteoporosis with vertebral collapse	None
4	Ray.	52	.12 .14	5.0 5.0	.90 2.10	2.50 5.90	.095 .253	1.0	Cystic lesion rt. 10th rib	None
5	Tar.	73	.08	10.0	1.25	5.60	.195	0.1	Normal	None
6	Mil.	80	.09	5.0	1.60	5.80	.266	—	—	None
7	Ree.	59	.15 .17 .29	10.0 5.0 5.0*	5.40 3.50 3.67*	12.20 6.99 4.16*	.423	3.1	Cystic lesion rt. 10th rib	None
8	Goo.	81	.05	5.3	3.63	23.70	1.110	4.4–2.8	Extensive Paget's disease	None
9	Ell.	73	.19	5.0	0.50	0.90	.041	3.6, 2.5, 1.6	Normal	None

* Frozen tissue used for testing.

reaction appears to be enzymatic in nature, since it is wholly abolished by boiling the tissues ten minutes prior to incubation. Furthermore, one metabolite (delta-4-androstenedione-3,17) has been regularly identified in the reaction products, on the basis of its chromatographic rate of migration and infrared spectrum. This compound has never been found during chromatographic analysis of control flasks.

In the early experiments there was considerable variation in the amounts of tissue, testosterone and buffer used. In spite of these variations in technic, different tissue specimens exhibit closely similar rates of steroid destruction, when the calculations are made on a per-cell basis, using desoxypentosenucleic acid (DNA) as a baseline. In the eighth case tested (Goo.) in view of the fact that there was some uncertainty about the clinical diagnosis, because of extensive bone disease and an asymmetrical hard prostate, an orchiectomy was performed. The serum acid phosphatase level may be elevated in Paget's disease, but in this patient the response of the serum acid and alkaline phosphatase levels following castration was more characteristic of prostatic carcinoma. In this benign group of cases there is always the possibility that a small carcinomatous focus missed by the pathologist was utilized for our experiments.

Examination of a series of pathologically proven prostatic carcinomas revealed a wider variation in the rates of testosterone metabolism (Table 3). Some specimens fell within the relatively low range of rates seen in benign tissues. In one of these, Case 2 (Hur.) the carcinomatous foci occupied only a small portion of the otherwise benign hypertrophic gland. Specimens of tissue from an anaplastic carcinoma present in a 38-year-old man (Case 1 (Sey.)) on two tests completely failed to cause any steroid destruction. The malignant growth in this case appeared similar to the other reported cases of prostate cancers occurring in young men (9). These cancers are usually anaplastic, produce little acid phosphatase, and seldom retain hormone sensitivity or dependency. In most of these cases the possibility existed that previous hormonal therapy had modified the response of the tumor cells to testosterone, but fortunately in the last case (No. 6, Cha.) with the highest rate of steroid degradation, this possibility was absent.

In Patient Ber. (Case 4), in whose tumor a moderately high rate of testosterone metabolism was present, excellent clinical evidence was obtained that orchiectomy and estrogens ameliorated his condition strikingly for a period of eighteen months. Testosterone administration caused marked exacerbation of pain in his osseous metastases, and he obtained a second subjective and objective remission lasting over eight months following total adrenalectomy.

In contrast, was Patient Haw. (Case 5), who showed a steady unremit-

TABLE 3. METABOLISM OF TESTOSTERONE BY PROSTATIC CARCINOMA

Case No.	Patient		Gm. tissue (dry wt.)	Mg. testosterone				Serum acid phosphatase (K.A.)	Bone roentgenograms	Endocrine therapy
	Name	Age		Added	Metabolized	Metab./Gm./hr.	Metab./mg. DNA/hr.			
1	Sey.	38	.60 1.26	20.0 20.0	0 0	0 0	0 0	4.5	No metastases	Estrogens
2	Hur.	61	1.90	16.8	12.8	2.29	.069	2.9	Lumbar spine metastases	None
3	Dow.	71	.23	10.1	3.05	8.82	.192	52.2	Generalized metastases	Estrogens
4	Ber.	58	.12	20.0	5.02	15.00	.711	34.5	Generalized metastases	Orchiectomy and estrogens
5	Haw.	52	.05	5.0	3.75	25.00	.890	50.0	Generalized metastases	Orchiectomy and estrogens; total adrenalectomy
6	Cha.	71	.07	5.0	4.30	21.10	1.300	2.9	No metastases	None

ting spread of his tumor in spite of orchiectomy, estrogens and adrenalectomy, and whose metastases at the present time possess somewhat higher rates of testosterone metabolism than those of Patient Ber. Patient Cha. (Case 6) showed the highest rate of steroid destruction, although there was no evidence that fifteen years of exposure to a normal male hormone environment in any way potentiated the growth of the tumor, which has not yet metastasized to bone.

DISCUSSION

Until now, it has been a matter of debate whether steroid hormones were actually metabolized by their specific target tissues. Attempts to demonstrate *in vivo* significant localization of testosterone labelled with radioactive carbon in animal target glands have not been successful (10). In view of our findings, there seems to be no question but that *in vitro* under the artificial conditions thereby imposed, rapid and extensive breakdown of testosterone occurs in this target gland, the prostate.

Involution of the normal adult prostate occurs soon after castration, indicating that testicular hormones exert a specific trophic function upon this organ (11). We have not yet encountered a specimen of benign hyperplasia of the prostate (which admittedly exhibits significant pathologic differences from the normal adult gland) that does not metabolize testosterone. With 1 exception, the tested prostatic cancers all metabolized

testosterone, often at rapid rates—a fact which may be significant in explaining the therapeutic value of castration in this disease. However, in at least 2 of our cases, rapid testosterone metabolism occurred in cancers which clinically did not appear responsive to androgenic stimuli or androgen deprivation. This suggests that further investigation is necessary concerning the rates of metabolism of adrenal androgens by these cancers, as well as the influence of various androgenic steroids upon protein synthesis in these tissues.

The rate of testosterone metabolism may be important in determining the tissue response to this hormone. A wide variety of body tissues undergo varying degrees of hypertrophy or hyperplasia under the influence of testosterone. Delta-4-androstenedione-3,17, produced by the enzymatic degradation of testosterone, has only one seventh the androgenic potency of the parent compound (12). In spite of uniform distribution of testosterone throughout the body by the vascular system, considerable variations in local concentration may occur, as shown by West in the rat and rabbit (13). No testosterone accumulation could be identified in either prostate or liver after intravenous administration, although a considerable amount was recovered from fat, muscle, and spleen. Over 75 per cent of the administered testosterone was cleared from the blood within ten minutes, even in hepatectomized nephrectomized animals, indicating a very rapid uptake of testosterone by the tissues. Accumulation could be explained by differences in the rapidity of enzymatic destruction of this steroid by the different tissues (14, 15). According to this interpretation, the elevated rates of testosterone metabolism seen in some of our cancer specimens indicate a probable reduction of sensitivity to stimulation by this compound through reduction of local concentration. These studies are being extended to other tissues besides male accessory sex organs, in order to gain further insight into factors altering the local tissue response to testosterone.

SUMMARY

Evidence is presented for a rapid enzymatic interaction *in vitro* between benign and malignant prostatic tumors and the prostate-trophic hormone, testosterone. Carcinomatous human prostate tends to metabolize testosterone more rapidly than does benign hypertrophic prostate. Carcinomas with rapid rates of testosterone metabolism may or may not respond clinically to androgens or androgen deprivation.

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USE OF DESOXYCORTICOSTERONE TRIMETHYLACETATE IN THE TREATMENT OF ADDISON'S DISEASE*

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IMPRESSIVE advances in the biochemistry and physiology of adrenal steroid hormones during the past quarter century have culminated in the development of a highly effective substitution therapy for adrenal cortical insufficiency (1, 2). The present report is concerned with a recent advance in the use of desoxycorticosterone. It has long been recognized that esters of desoxycorticosterone other than the acetate may exert a more prolonged action after intramuscular administration (3). Recently the trimethylacetate derivative (Fig. 1) has been prepared by Wieland *et al.* (4) and found to produce a protracted effect in animals following single injections of an oil solution. Preliminary studies in this clinic confirmed the latter effect (5). The treatment of patients with Addison's disease by intermittent intramuscular injections of desoxycorticosterone trimethylacetate (6, 7) was therefore undertaken.

MATRIALS AND METHODS

Studies have been carried out on patients with classic signs and symptoms of Addison's disease and a small group of subjects with carcinoma of

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the prostate who had been previously subjected to complete bilateral adrenalectomy. Patients were studied on the metabolism ward and in the out-patient endocrine clinic of the Peter Bent Brigham Hospital. Procedures utilized in the measurement of electrolyte balance, hematocrit, and blood urea nitrogen have been described previously (8). Heart size was determined from seven-foot postero-anterior chest films[1] taken during average inspiration; measurements were based on height-weight standards as described by Ungerleider (9).

Two esters of desoxycorticosterone were made available for clinical evaluation; desoxycorticosterone phenylacetate and desoxycorticosterone

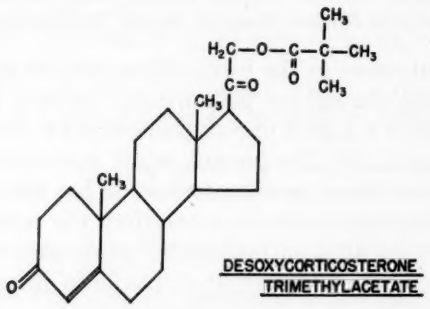

FIGURE 1

trimethylacetate[2]. The former preparation was administered for periods varying from two to twelve months to 16 patients with Addison's disease. Although a prolonged therapeutic action ranging from three to six weeks in duration was obtained, its effects were, in some instances, somewhat more abrupt and inconstant than those of the trimethylacetate. The latter agent was therefore selected for extensive clinical trial. The preparation used throughout these studies was a microcrystalline suspension of desoxycorticosterone trimethylacetate in isotonic saline, containing up to 1.3 per cent of methyl cellulose and 0.1 per cent of Tween 20 as suspending agents, and merthiolate (1:50,000) as preservative. An average crystal size of 50 micra (Fig. 2) insured a freely injectable suspension, the viscosity of which ranged between 14 and 26 centipoise.

During the past eighteen months 60 patients with chronic adrenal cortical insufficiency have been treated with desoxycorticosterone trimethyl-

[1] X-ray studies were made in the Department of Roentgenology, Peter Bent Brigham Hospital, under the direction of Dr. Merrill C. Sosman.

[2] All desoxycorticosterone preparations used in these studies were obtained from Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Inc., to whom we are greatly indebted.

acetate; 45 patients have been studied for a period of six months or longer and form the basis of this report. Prior to the initiation of treatment with the trimethylacetate ester, 37 of these patients had been maintained by repeated implantations of desoxycorticosterone acetate pellets; the remaining 8 had received daily injections of DCA in oil. Objective criteria employed for evaluating the state of hydration and electrolyte regulation included changes in body weight, blood pressure, hematocrit, blood urea nitrogen level, serum sodium/potassium ratio and heart size. The most informative index, both for patients and physicians, was the body-weight curve. A chart of daily weights, recorded on a printed form supplied to all

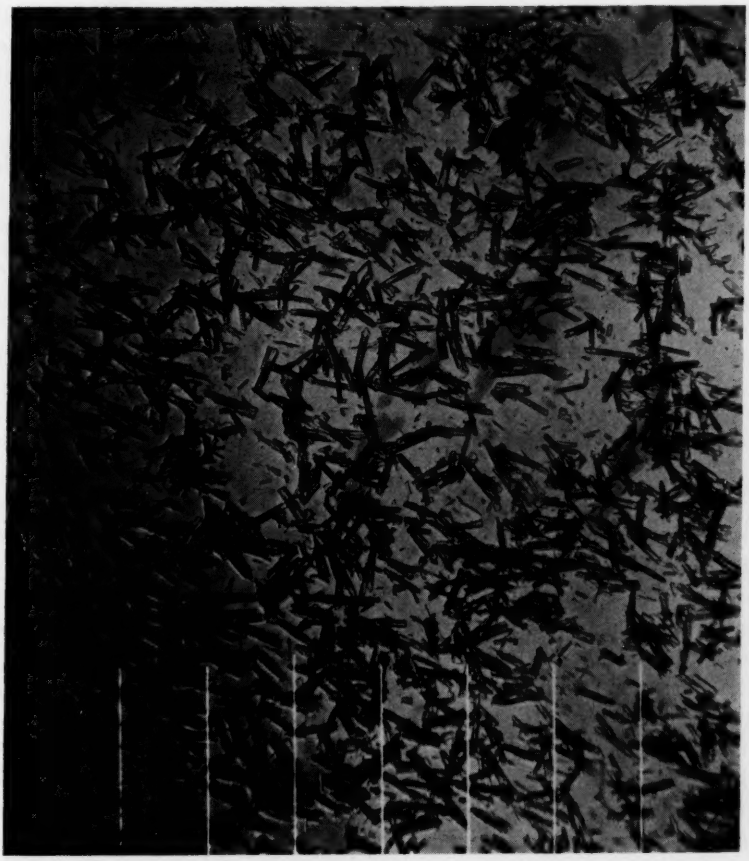

FIG. 2. Microphotograph of crystals of desoxycorticosterone trimethylacetate. Distance between vertical calibration lines: 100 micra. Magnification 125×. (Courtesy of Dr. C. H. Sullivan, Ciba Pharmaceutical Products, Inc.)

patients, was submitted at intervals of two to four weeks. This procedure proved exceedingly helpful in assessing both initial and maintenance dosage levels.

The majority of patients received supplementary oral cortisone therapy (12.5 to 25 mg. per day) during these studies. In order to facilitate the evaluation of changes in clinical status associated with the administration of desoxycorticosterone trimethylacetate, cortisone intake was maintained at a constant level unless the advent of an intercurrent infection or other complication required a temporary increase in dose.

OBSERVATIONS

The effects of desoxycorticosterone trimethylacetate on body weight, electrolyte excretion, hydration and cardiovascular function have been studied. However, the most important single criterion of the ultimate value of any agent submitted to therapeutic trial in chronic adrenocortical insufficiency has always been the clinical status of the patients treated, especially their capacity to withstand the varying demands of an active existence. Results of the routine use of this preparation in maintenance therapy in patients with chronic adrenal cortical insufficiency are therefore emphasized.

Body weight

One of the most sensitive indices of a change in the clinical condition of patients with Addison's disease is alteration in body weight. An improvement in clinical state is regularly accompanied by weight-gain; a relapse is usually presaged or accompanied by weight loss. As a corollary, a failure to gain weight signifies inadequate therapy; an excessive increase indicates over-treatment. Although the initiation of cortisone therapy results in a gradual, significant weight gain in nearly all patients, due mainly to a stimulation of appetite and increased food intake, more rapid fluctuations in body weight usually reflect alterations in mineral and water balance. Therefore, a continuing record of body weight is a useful means of assessing the results of desoxycorticosterone administration.

The initiation of desoxycortisosterone trimethylacetate therapy in untreated patients with Addison's disease or in those in whom previously implanted DCA pellets have been completely absorbed, is ordinarily associated with a significant gain in body weight. Figure 3 illustrates the changes observed at the beginning of desoxycorticosterone trimethylacetate treatment in 2 patients with Addison's disease who had been previously maintained by the implantation of desoxycorticosterone acetate pellets. Following the initial injection of trimethylacetate a definite gain in weight ensued, the maximum rise occurring during the first week. In those patients

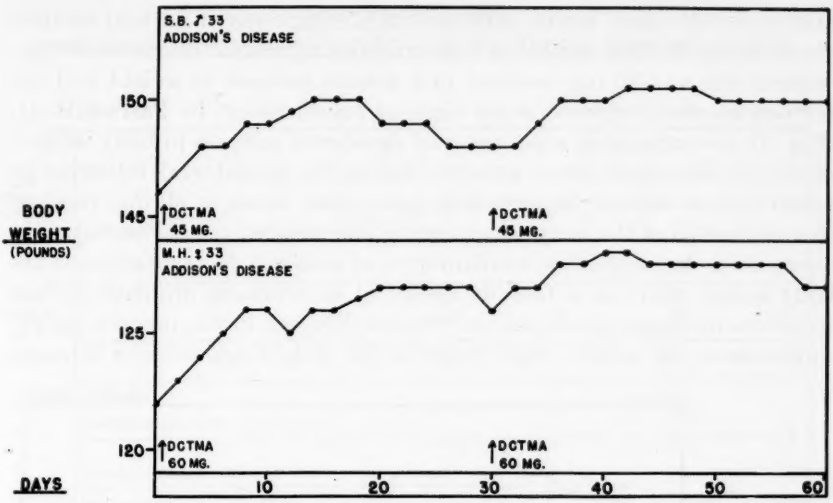

FIG. 3. Changes in body weight following initiation of desoxycorticosterone trimethylacetate therapy in patients with Addison's disease (previously maintained on desoxycorticosterone acetate pellets).

in whom the initial dosage had been correctly estimated (Fig. 3), subsequent injections produced less marked variations in body weight; indeed, the body-weight curve of many patients showed remarkably little change once long-term maintenance treatment had been established.

In some cases, however, adjustment of dosage was necessary in order to achieve ideal maintenance therapy. In Patient H. W. (Fig. 4) the maximum weight gain produced by the initial injection of desoxycorticosterone trimethylacetate (60 mg.) was somewhat less than that ordinarily observed;

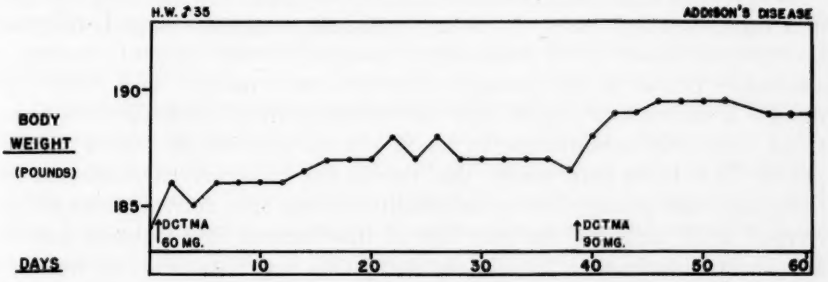

FIG. 4. Body-weight chart of patient, previously treated with desoxycorticosterone acetate pellets, in whom initial dose of desoxycorticosterone trimethylacetate was insufficient; subsequent increase in dosage produced adequate weight gain and symptomatic improvement.

approximately three weeks later moderate symptoms of adrenal cortical insufficiency (fatigue, weakness and anorexia) appeared. Elevation of subsequent doses to 90 mg. resulted in a greater increase in weight and the permanent disappearance of all signs of insufficiency. In Patient B. G. (Fig. 5), accompanying a prompt and significant increase in body weight, moderate dependent edema appeared during the second week following an initial dose of 60 mg.; reduction of subsequent doses to 30 mg. resulted in a depression of the body-weight curve and avoided the accumulation of excess fluid. Its simplicity, availability and economy have established the body-weight chart as a feasible means of determining optimum dosage schedules of desoxycorticosterone trimethylacetate in the manner shown. Furthermore, its use has contributed to the insight and security of many

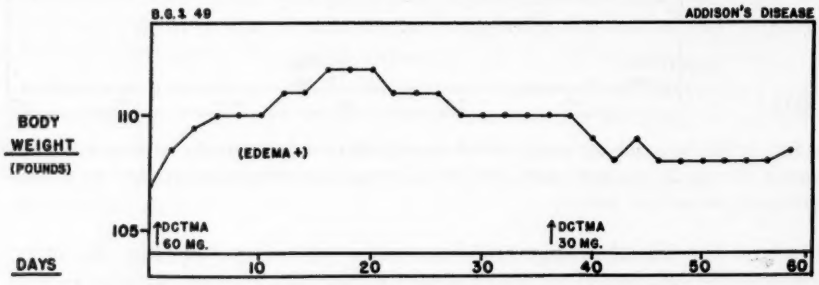

FIG. 5. Excessive weight gain and edema following the initial dose of desoxycorticosterone trimethylacetate in a patient with Addison's disease (previously maintained on desoxycorticosterone acetate pellets). Readjustment of dosage resulted in optimal control of weight and symptoms.

patients who, continuing voluntarily to keep daily records of body weight, have achieved a more confident regulation of salt intake and physical activity.

A representative curve of changes in body weight associated with the intermittent injection of desoxycorticosterone trimethylacetate during a six-month period of maintenance treatment in a patient with Addison's disease is shown in Figure 6. The variations recorded are little more than those ordinarily encountered in many normal individuals over a similar period. It is to be emphasized that during the half-year encompassed by the graph this patient led an essentially normal life, actively engaged in clerical work, and was entirely free of intercurrent infections or gastrointestinal disturbances. No dietary regulation was imposed. The freedom from hospital or dispensary care provided by such a therapeutic program is actually comparable to that achieved in the modern treatment of diabetes mellitus.

A composite of 100 weight charts collected from 35 patients, each chart

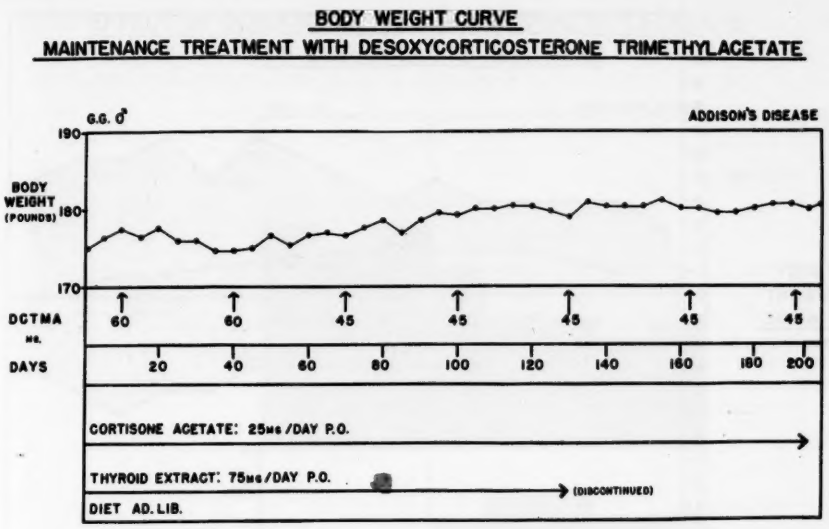

FIGURE 6

including a thirty-day period between injections, is shown in Figure 7. Both the maximum range of weight change and the mean body-weight curve on the days listed are depicted. The maximal limits of weight gain and weight loss recorded are an obvious reflection of overdosage and underdosage, respectively, and were obtained in all instances during the first two months of dosage regulation. Actually, 90 per cent of all graphs obtained after the first month showed a weight change of less than 5 pounds, and 80 per cent varied three pounds or less from the initial weight. Indeed, the range of variation was sufficiently narrow that any significant deviation from the mean may be taken as a reliable indication of either an erroneous dosage or an intervening complication.

Electrolyte excretion

The excessive urinary loss of sodium and chloride and concomitant retention of potassium occurring in patients with Addison's disease are well known. Desoxycorticosterone is the most potent agent at present available for the correction of these defects; indeed, at ordinary dosage levels, its action is confined almost entirely to the control of electrolyte excretion and distribution. The effects of desoxycorticosterone trimethylacetate have been studied by the technique of metabolic balance in 3 patients with Addison's disease. The results obtained were essentially similar in all cases. An example follows:

G.S., a 38-year-old female with Addison's disease of eleven years' duration entered

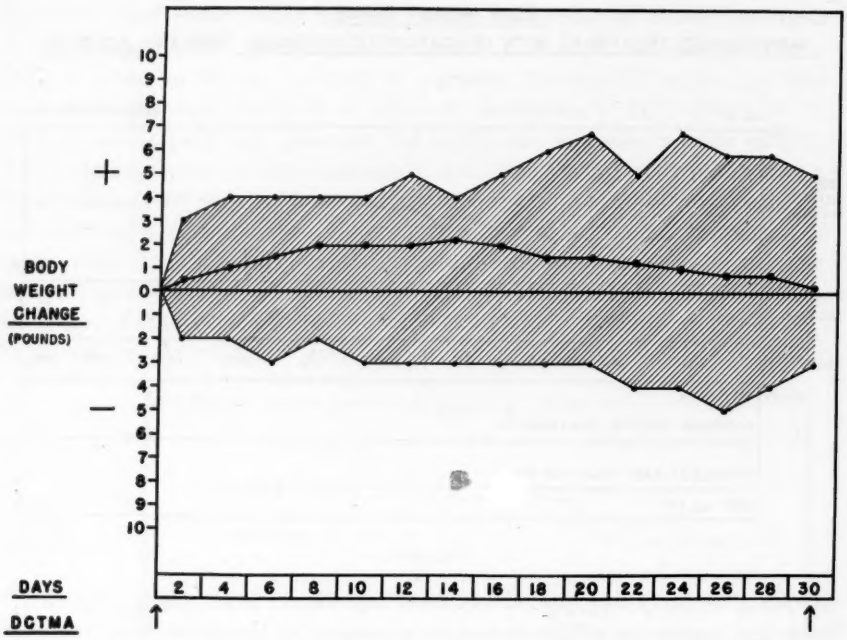

FIG. 7. Summary of 100 body-weight charts from 35 patients with Addison's disease receiving injections of desoxycorticosterone trimethylacetate at thirty-day intervals. Shaded area demarcates maximal changes in weight exhibited by the entire group; the mean body-weight change is also shown.

the Peter Bent Brigham Hospital two and one-half years following the last implantation of two desoxycorticosterone acetate pellets. Her sole therapy during the preceding twelve months had consisted of 25 mg. per day of oral cortisone acetate, which she continued to receive during the present study. Examination revealed an alert, somewhat dehydrated patient who complained of easy fatigability, muscular weakness, and persistent muscular cramps in the lower extremities. Blood pressure was 90/60 mm. of Hg. Following preliminary stabilization on a constant diet, a single 90-mg. dose of desoxycorticosterone trimethylacetate was injected intramuscularly. A significant retention of sodium and an increased urinary excretion of potassium occurred within twenty-four hours (Fig. 8). Muscular cramps were almost completely relieved within forty-eight hours.

It is apparent that the effects of desoxycorticosterone trimethylacetate on the renal excretion of electrolytes conform to the classic pattern established with desoxycorticosterone acetate. The trimethylacetate preparation attains its maximum effect within forty-eight to seventy-two hours, after which one may anticipate a prolonged period of relatively constant hormone action on electrolyte excretion. In most patients this effect is main-

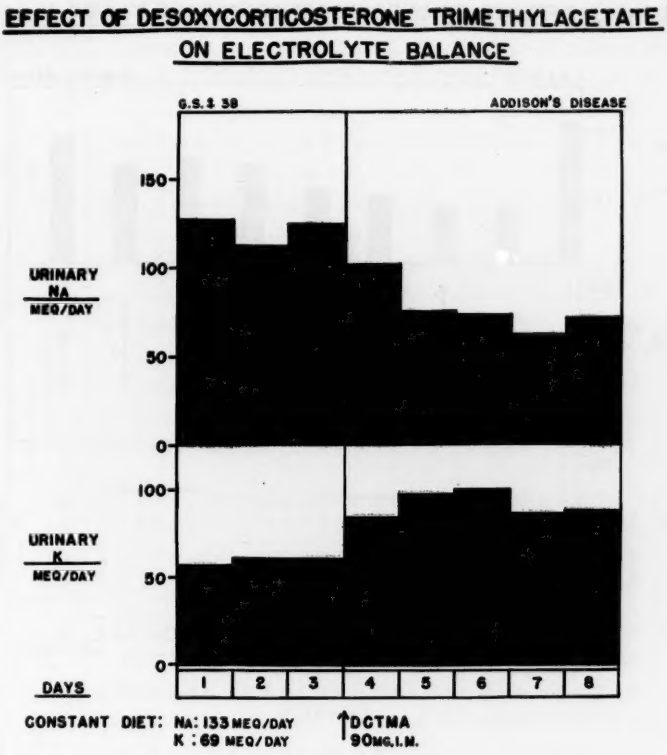

FIGURE 8

tained for a period of four to six weeks following the single intramuscular injection of the trimethylacetate ester of desoxycorticosterone.

Previous studies in this laboratory (10) have demonstrated the action of desoxycorticosterone on salivary electrolyte concentration. Measurement of the sodium/potassium ratio of this readily available fluid affords a relatively simple and rapid means of assessing significant alterations in the level of electrolyte-regulating adrenal cortical steroids. Figure 9 illustrates the effect produced by a single intramuscular injection of 60 mg. of desoxycorticosterone trimethylacetate in a patient with Addison's disease receiving a constant daily dose of cortisone acetate (12.5 mg. twice daily, by mouth); prior treatment with desoxycorticosterone acetate had been sporadic and inadequate, leading to obvious dehydration and salt depletion. A prolonged effect on electrolyte excretion is clearly evident, lasting approximately eight weeks and accompanied by a rise in blood pressure and a marked gain in body weight without the appearance of edema. Similar

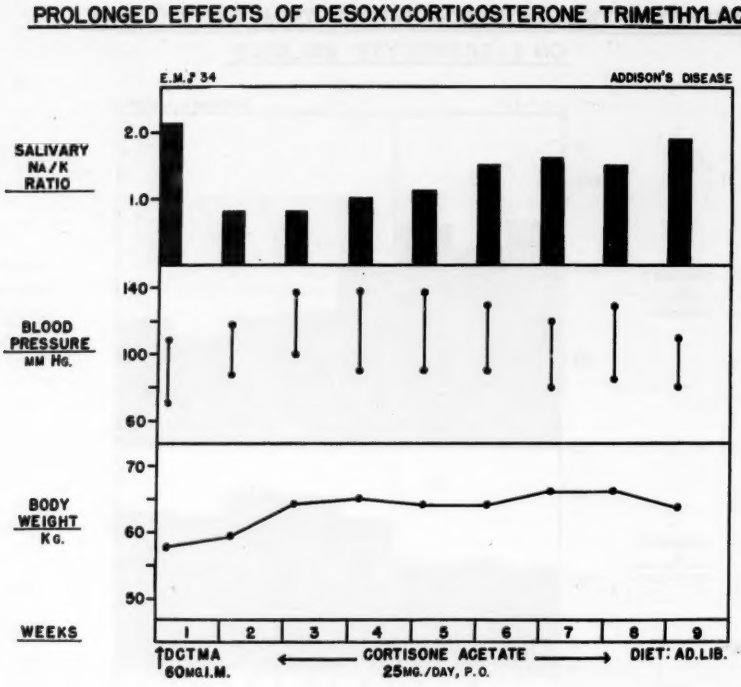

FIGURE 9

studies in other patients with adrenocortical insufficiency have demonstrated an average duration of effective electrolyte control of five to six weeks during routine maintenance therapy.

Hydration and cardiovascular function

The renal retention of sodium, chloride, and water which ensues from the administration of desoxycorticosterone to patients with adrenal cortical insufficiency results in increased plasma and extracellular volumes, accompanied by an elevation of blood pressure, expansion of cardiac volume and a rise in renal plasma flow and glomerular filtration rate. In addition to electrolyte balance and body weight, therefore, measurements of blood pressure, heart size, hematocrit and blood urea nitrogen provide valuable criteria for evaluating maintenance therapy. Table 1 records the results of treatment with the trimethyl ester of desoxycorticosterone in 28 patients with Addison's disease and 2 patients with carcinoma of the prostate previously subjected to orchiectomy and complete bilateral adrenalectomy. All of the patients included had been previously regulated on either pellets

TABLE 1. SUMMARY OF EFFECTS OF DESOXYCORTICOSTERONE TRIMETHYLACETATE IN ADDISON'S DISEASE

Patient	Age	Sex	Duration of disease (yrs.)	Previous DCA therapy	Duration of DCTMA therapy (mos.)	Dose of DCTMA (mg.)	DCTMA dosage interval (days)	Body weight (Kg.)		Blood pressure (mm. Hg)		Blood urea nitrogen (mg./100 ml.)		Hematocrit (%)		Serum sodium/potassium ratio		Heart size† (%)	
								Initial*	Present	Initial	Present	Initial	Present	Initial	Present	Initial	Present	Initial	Present
C.B.	20	F	4	2 Pellets‡	10	60	30	58	61	95/60	100/60	13	13	47	43	35	38	−30	−18
E.C.	26	M	4	2 Pellets	21	60	30	75	80	124/64	126/78	19	13	45	41	31	36	−20	−11
E.C.	18	M	6	3 Pellets	12	60	30	51	57	120/80	114/62	12	7	36	42	—	38	+15	+12
J.C.	37	F	2	DCA: 3 mg.	10	90	30	43	43	96/58	100/60	13	10	42	43	31	32	0	− 5
J.C.	55	M	18	2 Pellets	10	30	30	58	58	154/90	156/94	8	11	40	40	31	33	− 9	− 5
W.C.	36	M	19	2 Pellets	15	60	30	59	57	130/82	126/76	13	9	41	40	32	34	− 3	+ 2
C.D.	11	F	2	DCA: 2 mg.	12	45	30	25	28	92/66	88/66	25‖	22‖	40	38	32	38	—	—
W.D.	58	M	29	3 Pellets	12	60	30	63	64	118/68	144/84	16	7	41	40	34	39	− 7	+ 2
B.G.	51	F	4	2 Pellets	11	30	30	39	48	96/64	126/60	15	14	43	37	30	30	− 7	0
C.G.	52	M	9	4 Pellets	17	60	30	80	82	96/54	122/60	13	12	43	47	35	40	− 9	−10
D.G.	22	M	4	4 Pellets	17	60	30	78	86	104/70	124/78	17	14	39	41	29	31	− 2	−10
J.G.	41	F	5	2 Pellets	16	45	30	46	50	88/76	92/80	—	—	36	44	19	29	−11	0
J.H.	36	M	12	4 Pellets	16	60	30	70	70	130/90	130/86	12	13	49	47	31	32	− 6	− 7
K.H.	44	F	2	2 Pellets	10	60	30	60	58	102/80	142/84	12	13	43	47	32	36	0	0
L.H.	63	F	16	DCA: 3 mg.	8	45	30	45	53	96/66	116/60	16	13	33	37	28	33	− 5	− 4
N.L.	22	F	3	2 Pellets	11	60	30	68	67	116/76	120/80	14	8	43	44	32	32	−13	−16
H.McC.	48	M	13	6 Pellets	9	90	30	75	78	120/80	138/76	14	12	42	43	27	26	− 8	−12
N.McC.§	68	M	1	DCA: 2.5 mg.	11	60	30	59	61	150/86	152/88	11	12	38	38	29	29	0	− 3
J.McS.	25	M	1	DCA: 3 mg.	13	60	21	74	84	110/60	122/78	10	10	33	41	35	34	− 9	− 4
E.M.	48	F	6	2 Pellets	13	30	30	59	62	130/70	118/75	17	11	39	39	32	31	− 3	− 4
E.M.	34	M	3	3 Pellets	12	60	30	57	64	120/72	120/90	8	6	41	37	33	34	− 8	+ 4
N.M.	55	M	12	2 Pellets	13	30	30	70	74	110/70	130/98	13	9	46	47	30	32	+ 8	+ 5
M.N.	49	F	10	1 Pellet	12	15	14	69	70	126/84	128/80	17	12	42	44	29	28	−20	−12
T.P.	26	F	4	3 Pellets	16	90	30	46	48	98/66	100/72	8	8	38	42	35	36	− 8	− 5
O.R.	49	M	2	DCA: 2 mg.	14	60	30	77	83	102/65	132/92	11	11	35	38	34	32	− 7	− 7
D.R.	46	F	11	3 Pellets	13	90	30	66	67	130/70	138/76	22	13	36	38	32	34	− 5	− 7
C.S.	47	F	6	2 Pellets	18	60	30	49	55	115/70	138/82	6	9	37	39	31	33	−11	− 9
H.S.	44	M	5	3 Pellets	14	45	30	80	85	130/88	128/78	14	—	53	50	31	32	—	—
R.S.	53	F	10	2 Pellets	15	45	30	54	55	142/67	138/76	25	14	37	39	29	31	−15	− 4
W.S.§	76	M	1	DCA: 1 mg.	9	15	30	66	70	150/88	166/90	11	11	33	38	29	30	− 0	+12

* Values at time desoxycorticosterone trimethylacetate therapy was initiated.

† Heart size expressed as per cent predicted size derived from body-size data of Ungerleider and Clark (9).

‡ Desoxycorticosterone acetate pellets (Ciba), 125 mg., providing approximately 0.5 mg. of effective hormone per day.

‖ Values are for blood nonprotein nitrogen.

§ Patients with adrenal cortical insufficiency due to complete bilateral adrenalectomy; also, carcinoma of prostate and previous orchiectomy.

or daily injections of desoxycorticosterone acetate. The clinical condition of these patients at the time desoxycorticosterone trimethylacetate therapy was instituted was therefore generally satisfactory, although in some cases a sufficient period of time had elapsed since pellet implantation to permit the development of a moderate degree of dehydration and salt loss. The data presented adequately document the excellent control of hydration, electrolyte balance and cardiovascular function achieved in the maintenance treatment of Addison's disease with this agent. In no single instance have signs and symptoms of salt and water depletion developed in patients receiving desoxycorticosterone trimethylacetate at the recommended interval and in recommended dosage. Of the 13 male subjects listed, all but 2 (W. D., a retired seaman; and W. S., a 78-year-old tailor with carcinoma of the prostate and extensive bony metastases) are actively engaged in occupations ranging from accounting to carpentry; 6 of the female patients are employed in clerical or nursing positions. One patient, (H. R.) recently delivered a normal female infant following an uneventful pregnancy, during which maintenance hormonal therapy consisted of oral cortisone acetate (25 mg. per day) and 15 to 30 mg. of desoxycorticosterone trimethylacetate injected intramuscularly at thirty-day intervals.

Administration and dosage

Preliminary studies on the duration of action exerted by the trimethylacetate ester of desoxycorticosterone indicated the average period of readily demonstrable electrolyte regulation to be five to six weeks. Initially, therefore, injections were given at either five-week or six-week intervals, depending upon changes in body weight and blood pressure, and the physical capacity of the patient. It soon became evident, however, that weight loss, lassitude and decreased muscular efficiency often supervened during the period of one to two weeks preceding the next scheduled injection, an obvious reflection of inadequate absorption during this time. Elevation of dosage to prolong the period of effective control frequently resulted in the appearance of signs of over-treatment. An alternative method was therefore adopted, which has proved suitable for the great majority of patients: desoxycorticosterone trimethylacetate is administered by deep intramuscular injection, in a dosage calculated on the basis of the previously determined requirement of desoxycorticosterone acetate, at regular thirty-day intervals. The shortened period between injections produces a sufficient overlap of metabolic effect to avert all signs or symptoms of inadequate hormonal control.

Experience has demonstrated that approximately one-thirtieth to one-fiftieth of the injected dose is absorbed daily. Thus, administration of a single 30-mg. dose (1.0 ml.) results in a daily absorption of 0.6 to 1.0 mg.

of the material, accounting for a duration of activity ranging from four to eight weeks. In the majority of subjects, however, the period of effective hormonal action averages five to six weeks in length, pursuant to a daily release of approximately one-fortieth of the injected dose.

In initiating maintenance therapy with desoxycorticosterone trimethylacetate in a previously untreated patient, the principles of dosage regulation established for the use of desoxycorticosterone acetate pellets (1) are observed. A solution of DCA in oil is administered by daily intramuscular injection until a dose which is capable of maintaining a constant body weight is determined. For each milligram of desoxycorticosterone acetate solution injected daily, 30 mg. (1 ml.) of desoxycorticosterone trimethylacetate suspension is injected at thirty-day intervals. Although the quantity of active hormone absorbed each day from the intramuscular deposit of long-acting material is actually somewhat less than that supplied by daily injection of the acetate ester, the factor of continuous, constant absorption undoubtedly enhances the ultimate physiologic effect derived from the former, in the same manner as that described for desoxycorticosterone acetate pellets (11). On the other hand, a moderate prolongation of action beyond the arbitrary injection interval of thirty days is desirable, for the short overlap of activity derived from successive doses prevents the occurrence of sodium chloride and water loss during the final period of decreasing hormone absorption.

Patients maintained by DCA pellet implantation may be conveniently shifted to desoxycorticosterone trimethylacetate therapy at the time of incipient hormone deficiency resulting from the gradual decline in the quantity of daily hormone absorption. Estimation of dosage requires only a calculation of equivalent absorption ratios. Since one desoxycorticosterone acetate pellet (125 mg.) provides a daily hormonal effect equivalent to that obtained from the injection of 0.5 mg. of DCA in oil, 0.5 ml. (15 mg.) of desoxycorticosterone trimethylacetate suspension may be substituted for each pellet required. Since hormonal absorption from the suspension is undoubtedly somewhat more variable than from pellets, some patients may require a final dose of the long-acting suspension which is moderately greater than that calculated.

Prior to the initial implantation of desoxycorticosterone acetate pellets patients ordinarily undergo a variable period of regulation by injections of DCA in oil, in order that the proper pellet dosage may be ascertained. This period may usually be considerably shortened in determining the initial dose level of desoxycorticosterone trimethylacetate suspension because of a significantly shorter duration of action than that provided by subcutaneous pellets. A proper dosage adjustment for the long-acting suspension ordinarily requires only two, rarely three, monthly injections. This

increased flexibility of dosage manipulation constitutes a real advantage in its clinical use.

A summary of doses, administered at thirty-day intervals, utilized in the maintenance treatment of 45 patients with chronic adrenal cortical insufficiency is shown in Table 2. It is evident, on the basis of the dosage-

TABLE 2

DCTMA dose (mg.)	No. of patients
15	2
30	7
45	7
60	20
90	9

equivalents described, that the desoxycorticosterone requirement of the majority of patients ranges between 1 and 3 mg. per day, which is in agreement with previous observations (2).

Injections may be easily administered through a 21-gauge needle and are ordinarily painless and associated with no local signs of irritation. Although administration into the deltoid muscle is feasible, deep injection into the glutei is preferable in most cases.

Complications

The potential side-effects and overdosage phenomena of desoxycorticosterone trimethylacetate are those which may be encountered with any preparation of desoxycorticosterone and are the direct result of excessive salt and water retention and potassium elimination—excessive weight gain, edema, hypertension, hypokaliemia and eventually cardiac failure. Moderate, transient dependent edema has appeared in the first ten to fourteen days following the initial injection of a moderately excessive dose in some patients of this series. Subsequent dosage reduction, ordinarily in 15 to 30 mg. decrements, promptly eliminated this relatively minor complication. Three male patients in the older age group, J. C., W. S., and N. McC., had a moderately elevated blood pressure during the course of maintenance therapy with desoxycorticosterone trimethylacetate. In this type of individual, encountered more frequently during recent years due to the survival of many patients into the age of predilection for cardiovascular complications, a successful compromise can ordinarily be reached between the optimum dose for electrolyte control and that amount of hormone which permits the maintenance of a normal or only slightly elevated blood

pressure. Salt supplementation provides a simple and satisfactory means of maintaining balance in those occasional patients in whom a real reduction of hormone dosage becomes necessary.

During the course of approximately one thousand intramuscular injections of desoxycorticosterone trimethylacetate, four pyrogenic reactions occurred which must be attributed to this preparation. Within twenty-four hours following injection, these patients had a fever up to 103 degrees F., severe generalized malaise and headache accompanied by local redness, heat and pain at the site of injection. Because of the well-known susceptibility of patients with Addison's disease to noxious agents of all types, the prostration and fever accompanying these episodes required the administration of additional cortisone acetate to prevent the onset of adrenal crisis. Complete recovery spontaneously ensued within forty-eight hours in every case. An attempt to determine the responsible pyrogenic agent is currently under way in this and other laboratories.

DISCUSSION

Previous studies have delineated the important influence which both the physical state and form of esterification exert on the physiologic activity of steroids. Of the adrenal cortical steroids at present available, desoxycorticosterone has undergone the most intensive development. It is now available for therapeutic use in the following forms: (a) Desoxycorticosterone acetate solution in sesame oil for intramuscular injection; this preparation is ordinarily used for the initiation of desoxycorticosterone therapy and therefore provides the standard of reference for calculating dosage requirements of long-acting preparations. The duration of action exerted by a single injection is between twenty-four and forty-eight hours. (b) Desoxycorticosterone acetate in the form of tablets or propylene-glycol solution for intra-oral or sublingual use; adequate electrolyte control can be achieved with these preparations but approximately three to five times more hormone is required by this route than by intramuscular injection. The duration of action is transient and multiple intra-oral doses are necessary during each 24-hour period. (c) Desoxycorticosterone glucoside which, with the aid of acetamide, is sufficiently water-soluble to be used for intravenous injection. Although it produces a rapid onset of metabolic action its effects are transient and, in comparison with equivalent doses of the intramuscularly administered acetate ester, it is relatively impotent. (d) Desoxycorticosterone acetate pellets for subcutaneous implantation; this type of administration produces the longest effective therapeutic action (eight to twelve months) of the preparations currently available. It affords a reliable and well documented method of long-term maintenance therapy. (e) The aqueous suspension of desoxycorticosterone trimethylacetate for

intramuscular injection; recent studies in animals (12–14) have demonstrated unequivocally the protracted hormonal action exerted by this material. Its use in this study of 45 patients with chronic adrenocortical insufficiency demonstrates its effectiveness as a practical therapeutic agent.

The maintenance of body weight, blood pressure, hydration and cardiovascular function during continued treatment with this compound has been uniformly consistent. It is a pertinent fact that the great majority of patients in this series have indicated a definite preference for this method of desoxycorticosterone administration. Its advantages over other techniques of dosage are: (a) infrequent injections; (b) flexibility of dosage schedules; (c) avoidance of the minor surgical procedure involved in the implantation of pellets; and (d) constant control, especially in comparison with the final four to eight weeks of pellet therapy, during which time fatigability and muscular weakness are common. It is the authors' opinion that this technique of desoxycorticosterone administration currently constitutes the method of choice in the majority of patients.

SUMMARY

An aqueous, microcrystalline suspension of the trimethylacetate ester of desoxycorticosterone has been utilized in the maintenance treatment of 45 patients with chronic adrenal cortical insufficiency. Intramuscular injection of this compound produces effective electrolyte regulation, as demonstrated by metabolic balance studies, within twenty-four to forty-eight hours; the duration of action of a single intramuscular dose ranges from four to eight weeks, with an average duration of five to six weeks. Optimal therapeutic control, however, is obtained by injection of maintenance doses at thirty-day intervals. This schedule has been demonstrated to provide excellent control of body weight, blood pressure, hydration and cardiovascular function.

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THE CERVICAL MUCUS IN AMENORRHEA

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AS WE have already pointed out (1, 2) the first step in the study of a case of amenorrhea should be the establishment of its clinical type and degree; this is of fundamental importance for diagnosis as well as for treatment. Mild and severe amenorrheas may be similar clinically, but they are fundamentally different in their physiopathology. The essential difference lies in the presence or absence of estrogens, since the former manifest short single-phase cycles and in the latter the ovary has involuted.

The differential diagnosis is chiefly based upon the "functional examination," the ovarian activity being tested by colpocytology, the progesterone-test and endometrial biopsy. In the mild amenorrheas, these tests will show a trophic smear, a positive reaction, and proliferation, respectively.

In 1949 we stated (1) that physical exploration had very little importance in the determination of the clinical type of the amenorrhea. At that time, little or no significance was attached to the careful examination of the physical and microscopic characteristics of the cervical mucus, the study of which was carried out in detail only in cases of sterility.

The clinical importance of the cervical mucus has been recently proved, on checking its changes during the two-phase sexual cycle, in gestation, in castrates, during estrogen therapy (3, 4) and during a variable lapse of time after the menopause has occurred. From all this it can be concluded that there exists a close relationship between estrogens and cervical mucus, the latter being more abundant, transparent and fluid when the circulating estrogen concentration increases.

In 1948 Rydberg (5) showed that when a droplet of fluid transparent cervical mucus is smeared onto a microscope-slide and allowed to dry in air, crystallization in the form of fern leaves can be observed. These crystals do not appear or are much less noticeable when the mucus is scarce and turbid. Later (6) it was demonstrated that the crystals are produced from the combination of sodium-chloride and mucin.

In the same publication Rydberg (5) refers to observations on women with ovarian deficiency. Although he does not establish the clinical form

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of the deficiency, he recognizes that there is less cervical mucus present, and that the crystallization is atypical in many cases; he supposes that the mucus is poorer in sodium chloride under these circumstances.

In the two-phase sexual cycle, the crystallization of the mucus reaches its maximum, in form of fern leaves, during the ovulatory period.

Based upon these data and keeping in mind the already mentioned close relationship which exists between crystallization of mucus and concentration of estrogens, we felt it was important to study the macroscopic characteristics and the crystallization of the cervical mucus in mild amenorrheas, with the object of differentiating, by means of this simple and rapid procedure, mild amenorrheas from severe ones (*i.e.*, amenorrheas with and without estrogens).

TECHNIQUE AND MATERIAL

The technique is simple and within the scope of every physician who possesses a microscope. Once the speculum has been placed, the vagina and cervix are cleaned with gauze. If the external orifice is not too narrow, the end of a vaginal-dressing forceps is introduced and slightly opened and closed again within the cervical tract; it is then carefully withdrawn. In this way the cervical mucus is obtained, the viscosity or "Spinnbarkeit" of which is determined by slightly opening the two lips of the forceps and measuring the length of the filament formed, in centimeters. Then the mucus is smeared onto a microscope-slide and left to dry in air, or is slightly heated. When the vaginal orifice is narrow, the endocervical mucus is

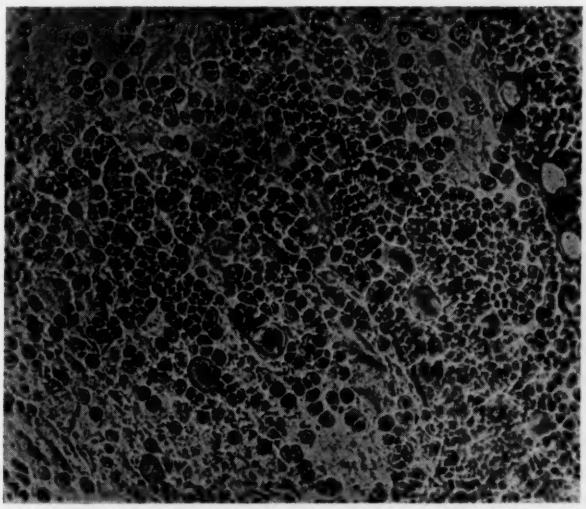

FIGURE 1

sucked up with a thin well-dried pipette connected to a syringe or a rubber bulb and discharged onto a slide. When carrying out this procedure, care has to be taken to avoid drawing blood, since when this occurs, it disturbs the crystallization and the test has no diagnostic value.

The physical characteristics of the mucus are determined and recorded with regard to:

1. Quantity
2. Transparency
3. Fluidity and viscosity

Five minutes are sufficient for observing the mucus by microscope with an enlargement of 120 diameters. The result of the test is *negative* (Fig. 1) when

FIGURE 2

only cell groupings can be seen. The result is *positive* (Fig. 2) when crystallized dispositions are seen in the form of large sized fern leaves, similar to those of Samambaia or Brazilian Selaginella, as stated by Campos da Paz (4). In other instances, the crystallization is not so distinct (Fig. 3) and has the appearance of Sawfern or of Nandutí tissues; these forms occur only in certain areas of the smear and are considered atypical.

The cervical mucus has been studied in 28 patients with amenorrhea lasting from forty days to fifteen years; in nearly all of them the degree and the clinical form of the amenorrhea were determined by means of the progesterone test; in some of them biopsy of the endometrium and the colpocy-

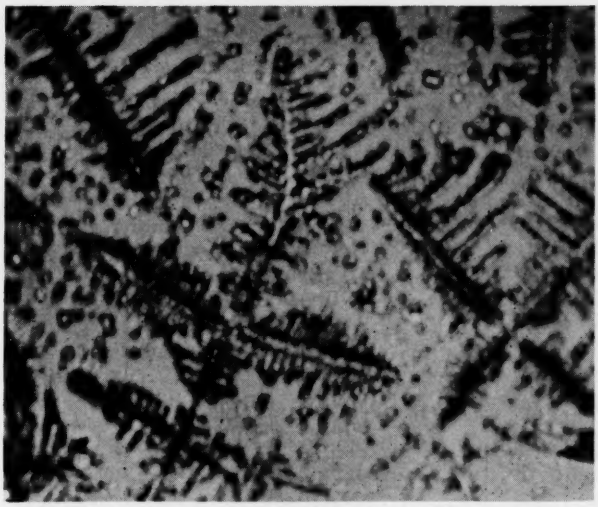

FIGURE 3

tologic study were performed. In 11 cases of amenorrhea (Table 1) thus individualized, the scarcity of cervical mucus and absence of crystallization were proved; in all these, administering estrogens caused the appearance of a fluid and transparent mucus with intense crystallization.

TABLE 1

Case	Age	Amenorrhea		Results of			State of cervix
		Mild	Severe	Mucus cryst. ("fern") test	Endometrial biopsy	Progesterone test	
1	22	—	yes	Negative	Negative twice	Negative	Healthy
2	37	—	yes	Negative	Hypotrophic (Shorr)	Negative	Healthy
3	36	—	yes	Negative	—	Negative	Healthy
4	27	—	yes	Negative	Atrophic (Shorr)	Negative	Healthy
5	32	—	yes	Negative	Negative	Negative	Healthy
6	25	—	yes	Negative	—	Negative	Healthy
7	41	—	yes	Negative	—	—	Healthy
8	28	—	yes	Negative	—	Negative	Healthy
9	42	—	yes	Negative	Negative	Negative	Healthy
10	30	—	yes	Negative	Surg. castrate	—	Healthy
11	27	—	yes	Negative	Roentg. castrate	—	Healthy

TABLE 2

Case	Age	Amenorrhea		Results of			State of cervix
		Mild	Severe	Mucus cryst. ("fern") test	Endometrial biopsy	Progesterone test	
1	35	yes	—	Positive	Proliferation	Positive	Healthy
2	32	yes	—	Atypical	—	Positive	Cervicitis
3	40	yes	—	Positive	—	Positive	Healthy
4	24	yes	—	Positive	Proliferation	Positive	Healthy
5	35	yes	—	Positive	Proliferation	—	Healthy
6	31	yes	—	Positive	—	Positive	Healthy
7	24	yes	—	Positive	Proliferation	Positive	Healthy
8	30	yes	—	Positive	Proliferation	—	Healthy
9	32	yes	—	Positive	—	Positive	Healthy
10	28	yes	—	Positive	—	Positive	Healthy
11	36	yes	—	Negative	—	Positive	Healthy
12	45	yes	—	Positive	—	Positive	Healthy
13	52	yes	—	Positive	—	Positive	Healthy
14	39	yes	—	Positive	—	Positive	Healthy
15	52	yes	—	Positive	—	Positive	Healthy
16	31	yes	—	Positive	—	Positive	Healthy
17	21	?	?	Positive	—	Negative	Healthy

In 17 mild cases (Table 2) the presence of transparent mucus was observed, which clearly crystallized in 14 instances. It was atypical in 1 case which also exhibited an endocervicitis. In another case, the result of a progesterone test was positive and the mucus did not crystallize. In the remaining case, the result of a progesterone test was negative and the mucus crystallized; in this instance there was a subsequent trial of estrogens, in order to eliminate an uterine amenorrhea.

COMMENT

This study clearly proves that in mild amenorrheas in which ovarian activity is partially preserved in the form of short single-phase cycles, the mucus crystallization test ("fern test") gives positive results in nearly all the cases. On the contrary, in severe cases of amenorrhea in which ovarian deficiency is total, no crystallization takes place; it appears only after the administration of estrogens.

These findings suggest that this test provides another useful element for the efficient diagnosis of the clinical form of amenorrhea. It thus helps, together with the progesterone test, colpocytology and endometrial biopsy,

TABLE 3

Clinical condition	Crystallization of cervical mucus
First half of cycle	+
Second half of cycle	−
Gestation	−
Mild amenorrhea	+
Severe amenorrhea	−
Castrates	−
Estrogen therapy	+

to complete the functional examination with regard to the degree of ovarian activity, and has the advantage of speed and simplicity of method.

It should be remembered that the increased amount, fluidity and transparency of the mucus and its crystallization are observed in the first half of the two-phase sexual cycle, in the single-phase cycles, in mild amenorrheas and during treatment with estrogens. The mucus is absent or is scarce and does not crystallize in castrates and in severe amenorrheas, *i.e.*, when there are no estrogens. The mucus is scarce or pale and more viscous and does not crystallize in the second half of the two-phase sexual cycle, and in the beginning of gestation, *i.e.*, when progesterone is acting (Fig. 3).

SUMMARY

The application of the cervical mucus crystallization test to the diagnosis of amenorrheas, is presented. This test gives positive results in mild amenorrheas and negative results in severe amenorrheas.

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SERUM CONCENTRATIONS OF RADIOIODINE IN DIAGNOSTIC TRACER STUDIES*

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IN RECENT years radioactive iodine, I^{131}, has been utilized widely in the diagnosis and study of thyroid disease (1). In spite of this, relatively little attention has been given to the measurement of serum radioiodine concentrations in patients receiving tracer amounts of I^{131} for routine diagnostic studies; particularly is this true of the study of serum I^{131} concentrations at periods of four days and longer following the administration of radioiodine. Recently Silver and Fieber (2) have described the results of such measurements and their usefulness in the diagnosis of hyperthyroidism. The present work provides confirmation of the results of these authors and further extends the application of this procedure in the study of thyroid metabolism.

METHODS

Tracer studies for determination of thyroid status are carried out routinely by the Radioisotope Laboratory at the request of the various clinical services of the hospital.

On the morning of the test 50 microcuries of I^{131} are given by mouth in about 100 ml. of water; patients are not required to be in the fasting state. Thyroid uptake of radioiodine is measured at 6 hours and 24 hours, using a shielded end-window counter. Two urine specimens, 0–24 hours and 24–48 hours, are obtained routinely; in some cases an additional 24-hour collection, 48–72 hours, is made. By careful instruction of the individual patient with the assistance of the nursing staff every effort is made to insure completeness of urine specimens. Percentage urinary excretion is determined by simultaneous comparison of radioactivity of aliquots of urine and tracer solution, by use of

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* Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

The radioactive iodine used in this investigation was supplied by Oak Ridge National Laboratory on authorization from the Isotopes Division, U. S. Atomic Energy Commission.

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Marinelli beakers and a glass-wall gamma-sensitive Geiger tube. Blood specimens, obtained by venipuncture, are collected ordinarily one day and either four, five or six days after administration of radioiodine. In some cases additional specimens at longer intervals after beginning the test are obtained. After clotting, the serum is separated by centrifugation and duplicate 1-ml. aliquots are pipetted on aluminum planchets ($1\frac{1}{32}$ inch diameter; 10 square centimeters area) containing a few drops of wetting agent (1 per cent sodium lauryl sulfate). After drying slowly with an overhead infrared lamp the planchets are counted in a windowless methane-flow proportional counter (Nucleometer, Radiation Counter Laboratories, Chicago, Illinois). Results are expressed in percentage of administered radioiodine per milliliter of serum without making any correction for the 9 mg./cm.^{2} serum self-absorption. These values may be converted to absolute percentage by use of a multiplying factor, 1.52, experimentally determined under the conditions described.

RESULTS

A. *Serum radioiodine levels of patients in routine tracer studies*

Serum radioiodine concentrations were measured in 44 patients. On the basis of clinical observation, thyroid uptake and urinary excretion of radioiodine, these were classified as normal, hypothyroid or hyperthyroid. In this limited series no patients were encountered about whom there existed reasonable doubts as to the proper classification category. Of primary importance in this study was the establishment of the range of serum radioiodine concentrations in normal individuals. For this purpose tracer studies on the group of 34 euthyroid individuals, obtained in the course of routine diagnostic tracer studies, were supplemented by tracer studies on 40 additional convalescent patients from the medical service. The clinical diagnoses for this group of 40 were as follows: tuberculous peritonitis, 1; infectious hepatitis, 2; pulmonary disease, 5; peptic ulcer, 9; and cirrhosis of the liver, 23.

Urinary excretion for 48 hours and serum levels of radioiodine at one and four days were measured as described. All patients were clinically euthyroid; urinary excretion of radioiodine in each case was normal—range 34 to 80 per cent. Serum concentrations of radioiodine for this combined group of 74 euthyroid individuals are plotted as a function of time in Figure 1. Similarly serum levels of 9 hyperthyroid patients are plotted individually in the same figure. The single patient with hypothyroidism is not included, as this condition does not appear to be differentiable from normal thyroid function by serum radioiodine measurements made in this manner.

For the purpose of comparing serum radioiodine concentrations in different patients it is desirable to devise a single measure of the variation of radioactivity with time. A desirable characteristic of such a measure is that the transformed data shall be normally distributed. This may be achieved for each patient by the computation of the following statistic:

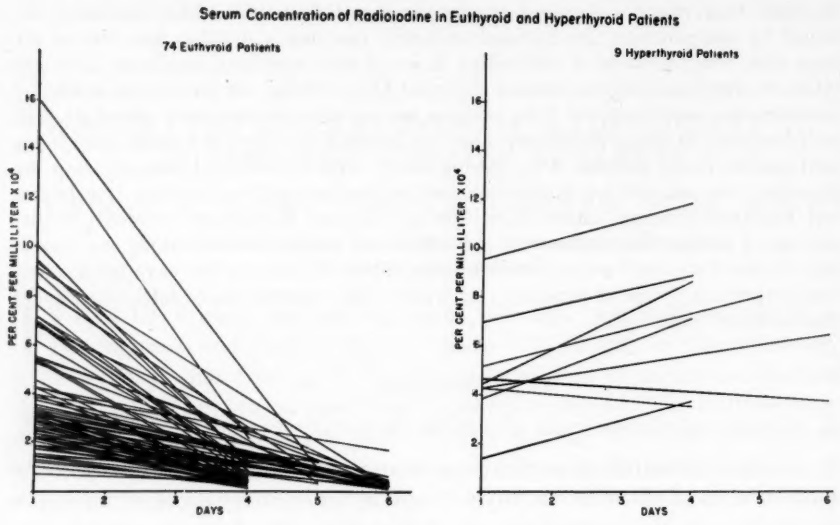
Serum Concentration of Radioiodine in Euthyroid and Hyperthyroid Patients
74 Euthyroid Patients
9 Hyperthyroid Patients
PER CENT PER MILLILITER X 10⁴
DAYS

FIGURE 1

$$i = \log_{10}\left[\frac{(P_4)^2}{P_1} \times 10^7\right]$$

where P_1 is the serum radioiodine concentration per milliliter in percentage of administered radioactivity at one day; and P_4 is the same value at four, five or six days. This statistic was calculated for each of the patients in Figure 1, and a frequency distribution of the results is plotted in Figure 2.

The distribution of i for the 74 euthyroid patients, which appears to be normal, was tested for agreement with the normal distribution by calculation of the statistics g_1 and g_2 (3). The statistic g_1 is a measure of the symmetry with which values are grouped positively and negatively about the mean; g_2 measures conformity with the normal distribution with respect to flatness or peakedness of the curve. Both are equal to 0 for the normal distribution. In this case neither g_1 nor g_2 differs significantly from 0:

$$g_1 = 0.088,\ t = 0.32,\ p > 0.5$$
$$g_2 = 0.31,\ \ t = 0.56,\ p > 0.5$$

This permits the calculation of 1 per cent confidence limits (4) for i for the mean of the euthyroid individuals, namely 1.64 ± 1.80. A patient with a a value of i greater than 3.44 may be considered to be not euthyroid, inasmuch as there are only two chances in a hundred of an euthyroid individual having such a value of i.

It should be noted, as is pointed out under Methods, that inasmuch as the percentages of the administered dose per milliliter of serum values have

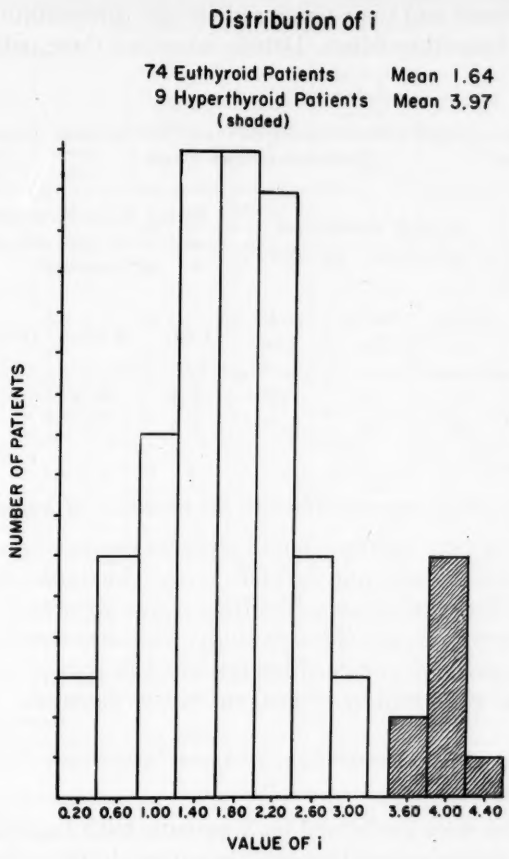
Distribution of i
74 Euthyroid Patients Mean 1.64
9 Hyperthyroid Patients Mean 3.97
(shaded)
NUMBER OF PATIENTS
0.20 0.60 1.00 1.40 1.80 2.20 2.60 3.00 3.60 4.00 4.40
VALUE OF i

FIGURE 2

not been corrected for the self-absorption of serum it is necessary to add a constant, $\log_{10} 1.52$ or 0.18, to each value of i in order to express i in absolute terms. Similarly 0.18 should be added to the mean of i and the confidence limits in order to express them in absolute values. For comparison purposes within any one series, it is not necessary to make these corrections.

None of the 9 hyperthyroid patients had a value of i falling within the normal range, as is seen in Figure 2. The significance of the difference between the means of the hyperthyroid and euthyroid groups was tested by "Student's" t (5):

$$t = 18.9,\ p < 0.01$$

Consequently this is a real difference.

Values of i for 2 patients with cirrhosis of the liver were also significantly

greater than normal and their serum radioiodine concentrations were similar to those in hyperthyroidism. Details regarding these patients are seen in Table 1.

TABLE 1. SERUM RADIOIODINE CONCENTRATIONS IN 2 CASES OF CIRRHOSIS OF THE LIVER

Patient	Urinary excretion of radioiodine: per cent			Serum radioiodine concentration: per cent per milliliter × 10^4			i
	0–24 hrs.	24–48 hrs.	0–48 hrs.	1 day	4 days	16 days	
FS #29016	64	8	72	5.8	16.0	0.7	4.64
WG #33215	48	3	51	1.3	2.7	—	3.75

B. *Serum radioiodine concentrations in the treatment of hyperthyroidism*

Tracer studies were performed on 5 patients during the course of therapy by radioiodine or following thyroidectomy. The results are summarized in Table 2. Serum radioiodine concentration was apparently a good index of clinical status at the time of tracer study, with the exception of 1 patient (LS) whose serum level remained hyperthyroid in spite of the fact that at the time he was euthyroid by clinical and tracer diagnosis.

C. *Serum radioiodine concentrations in hyperthyroidism treated with propylthiouracil* (Table 3)

Tracer studies were performed on 3 patients with hyperthyroidism before, during and after propylthiouracil therapy with 300 milligrams per day. Following an initial tracer study, each patient received propylthiouracil treatment for fifteen to twenty-two days, during which time repeat tracer studies were performed; Patient DE received a second tracer on the twelfth day of antithyroid therapy; Patients CT and JB, on the eighth day. Therapy was continued for twenty-two days in Patient DE and for fifteen days in Patients CT and JB. In each case a third tracer study was performed two days after discontinuing propylthiouracil treatment.

D. *Duration of serum radioiodine concentrations in hyperthyroidism* (Table 4)

In addition to measurements at one and four, five or six days, which were made on all patients, serum radioiodine concentrations were determined in 6 hyperthyroid patients at various times up to sixteen days.

TABLE 2. SERUM RADIOIODINE CONCENTRATIONS IN THE TREATMENT OF HYPERTHYROIDISM

Patient	Therapy and date	Tracer study date	Thyroid uptake %		Urinary excretion %		Serum I^{131} concentration: per cent per milliliter $\times 10^4$		i	Tracer diagnosis†	Clinical diagnosis at time of tracer
			6 hrs.	24 hrs.	0–24 hrs.	24–48 hrs.	1 day	4 days			
DE #30544	None*	Aug. 27, '51	32	42	36	2	3.8	7.2	4.13	Hyperthyroid	Hyperthyroid
	5 mc. I^{131} Oct. 25, '51	Jan. 10, '52	10	19	47	—	3.8	3.2	3.43	Euthyroid	Euthyroid
WW #30841	4.7 mc. I^{131} Mar. 6, '51	May 3, '51	15	19	43	5	2.2	1.3	2.89	Euthyroid	Euthyroid
	—	Sept. 17, '51	60	58	11	—	4.5	8.8	4.24	Hyperthyroid	Hyperthyroid
FB #30250	6.5 mc. I^{131} Oct. 2, '50	July 31, '51	25	37	32	2	4.6	3.8‡	3.50	Hyperthyroid	Hyperthyroid
	28 mc. I^{131} Aug. 30, '51	Dec. 4, '51	3	2	69	14	4.7	0.3‡	1.28	Hypothyroid	Hypothyroid
	—	Apr. 9, '52	4	6	38	35	6.9	0.5§	1.56	Hypothyroid	Hypothyroid
	—	July 22, '52	5	7	57	27¶	7.0	0.3‡	1.08	Hypothyroid	Hypothyroid
LS #30052	T'y, 1946‖	July 9, '51	—	27	36	—	4.3	7.8	4.15	Euthyroid	Euthyroid
GM #32718	T'y, 1947‖	Feb. 11, '52	31	45	47	1	0.8	1.0	3.10	?Hyperthyroid#	Euthyroid

* Prior to initiation of therapy.
† Based only on urinary excretions and thyroid uptake.
‡ Six-day serum.
§ Five-day serum.
¶ Includes 8% 48–72 hrs. excretion.
‖ Subtotal thyroidectomy.
Thyroid uptake, hyperthyroid; excretion, normal.

DISCUSSION

A general problem in the validation of any test of thyroid function which proposes to classify individuals into definite categories concerns the necessity of finding criteria of classification independent of the test in question. Furthermore these criteria should be of such a nature as to permit the unequivocal classification of each individual who has been studied by the proposed test. With respect to determination of thyroid status the greatest difficulty frequently is encountered in attempting to decide fairly whether certain individuals should be classified as euthyroid or hyperthyroid. This problem may sometimes be resolved by the creation of the additional category of mild hyperthyroidism. However, the most useful test of thyroid function from the clinical point of view would be one which could predict

TABLE 3. SERUM RADIOIODINE CONCENTRATIONS AND PROPYLTHIOURACIL TREATMENT

Patient	Tracer study	Urinary excretion of radioiodine (%)			Serum radioiodine concentration(S): per cent per milliliter×10^4. Thyroid uptake (T): per cent				*i*
					1 day		4 days		
		0–24 hrs.	24–48 hrs.	0–48 hrs.	S	T	S	T	
DE #30544	Initial*	36	2	38	3.8	42	7.2	—	4.13
	Propyl*	57	7	64	2.9	8	1.1	7	2.62
	Post-propyl*	33	4	37	2.8	46	5.8	37	4.08
CT #32448	Initial	23	3	26	1.4	71	3.8	—	4.01
	Propyl	62	14	76	5.3	30	1.0	23	2.27
	Post-propyl	25	—	—	1.5	73	2.3	—	3.55
JB #32881	Initial	17	1	18	7.0	49	9.0	—	4.10
	Propyl	43	10	53	4.8	27	4.1	14	3.54
	Post-propyl	13	—	—	19.0	38	20.0	—	4.32

* See text for details.

accurately a favorable response to specific thyroid therapy in patients suspected of hyperthyroidism. Within the limits of the small number of hyperthyroid patients included in this study, serum radioiodine concentration measurements appear to have been successful in this regard. Each patient in the hyperthyroid group received specific thyroid therapy, either thyroidectomy or radioiodine, with subsequent clinical improvement. This approach to the problem of classification and validation will be emphasized in future studies of serum radioiodine concentrations in this laboratory.

TABLE 4. DURATION OF SERUM RADIOIODINE CONCENTRATIONS IN HYPERTHYROIDISM

Patient	Serum radioiodine concentration: per cent per milliliter×10^4			
	1 day	4–6 days	7–9 days	13–16 days
JB #32881	7.0	9.0 (4 days)	9.2 (7 days)	—
CT #32448	1.4	3.8 (4 days)	3.3 (7 days)	—
JB #615692	—	7.4 (6 days)	8.8 (9 days)	—
VR #33044	0.6	—	—	2.2 (13 days)
CB #30938	5.2	8.2 (5 days)	—	5.1 (15 days)
HM #30926	1.1	—	—	1.4 (16 days)

Further evidence concerning the relationship of serum radioiodine levels to clinical status and their modification by therapy is provided by the group of hyperthyroid patients studied during the course of treatment by thyroidectomy or radioiodine. Generally there is a close parallel between the serum radioiodine concentration and the status of the patient as determined by clinical and tracer study. The persistence of a high serum radioiodine concentration in 1 thyroidectomized patient is of considerable interest. This patient was clinically euthyroid and had a normal thyroid uptake and urinary excretion of radioiodine at the time of tracer study. It is possible that serum radioiodine levels frequently do not revert to normal following therapy of hyperthyroidism. This point is under further study.

The significance of high serum radioiodine levels in the 2 patients with severe cirrhosis of the liver is not clear at this time. There was in these individuals no suggestion of hyperthyroidism, either on the basis of urinary excretion of radioiodine or clinical observation. Other cases of cirrhosis did not show this behavior; to what extent this phenomenon is related to liver disease or to some other condition warrants further investigation.

The effectiveness of propylthiouracil in modifying simultaneously the serum radioiodine concentration and the thyroid uptake and urinary excretion of radioiodine in hyperthyroidism is suggestive of the probable nature in this disorder of the serum component containing radioiodine. The action of propylthiouracil in hyperthyroidism has been ascribed to an interference with the production of thyroid hormone (6, 7). The behavior of serum radioiodine concentrations in hyperthyroidism under the influence of propylthiouracil is consistent with the interpretation that an elevated four-day level of serum radioiodine in this disease is due to increased circulating thyroid hormone.

SUMMARY

1. Serum radioiodine concentrations one and four days after oral administration of 50-microcurie tracer doses have been studied in 74 euthyroid and 9 hyperthyroid patients.

2. An index of the serum radioiodine level, *i*, has been described and its usefulness in the diagnosis and study of thyroid disease discussed.

3. Evidence is presented which suggests that higher than normal serum radioiodine concentrations in hyperthyroidism at four days and longer after the administration of tracer doses represent iodine incorporated into circulating thyroid hormone.

Acknowledgment

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Letters to the Editor

HIGH PBI^{131} CONCENTRATION IN BLOOD OF PATIENTS WITH MYXEDEMA: PRELIMINARY REPORT

To The Editor:

The rate of appearance of organically bound I^{131} in the blood is generally considered as an index of thyroid gland activity. Recently, however, we observed 2 cases with clearcut hypothyroidism in which the concentration of PBI^{131} rose to abnormally high values.

CASES

Case 1. A female patient, aged 52 years, had hyperthyroidism in 1951, and in January 1952 a subtotal thyroidectomy was performed. In October 1952 sensitivity to cold and failing memory indicated hypothyroidism. At examination she showed a dry skin, a pulse rate of 56 per min. and BMR −20 per cent. The serum concentration of cholesterol was 312 mg. per 100 ml. and of PBI, 1.3 μg. per 100 ml.

Case 2. A female patient, aged 57 years, underwent subtotal thyroidectomy for hyperthyroidism in 1943. In 1952 she was seen for complaints that were already of long standing: fatigue, sensitivity to cold, and failing memory. At examination she showed a dry skin, a pulse rate of 60 per min. and BMR −16 per cent. The serum concentration of cholesterol was 475 mg. per 100 ml. and of PBI, 1.3 μg. per 100 ml. The electrocardiogram showed low voltage with typical T-wave changes.

Treatment with 100 mg. of desiccated thyroid daily was successful.

METHODS

In both patients I^{131} studies were carried out (Table 1); in Case 2 these studies were repeated, and the results showed good agreement with those first obtained.

Determinations of PBI^{131} were made in 10 per cent trichloroacetic acid precipitates, which were washed three times with 1 per cent trichloroacetic acid. Elutability of iodide was tested by adding I^{127} and I^{131} in vitro to several serum specimens from Case 2; it was shown to be at least 94 per cent. Therefore nonelutability of iodide can only account for a conversion ratio of 6 per cent at most. Moreover this explanation is to be discarded because of the rising PBI^{131} concentrations with simultaneously decreasing radioiodide concentrations.

Quite unexpected were the results of the PBI^{131} determinations. Definite proof that the observations were correct was obtained by graphic analysis.

TABLE 1

	Urine (% of dose excreted)	Thyroidal uptake (% dose)	Serum		Conversion ratio* (%)
			Total activity (% dose/liter) (A)	Protein-bound activity (% dose/liter) (B)	
Case 1					
24 hrs.	57.9	21	0.620	0.254	41
48 hrs.	63.9	22	0.600	0.426	71
Case 2					
Test I:					
24 hrs.	71.7	7	1.070	0.101	9
48 hrs.	86.8	7	0.370	0.192	52
72 hrs.	—	6	0.305	0.268	88
Test II:					
24 hrs.	69.7	3	0.872	0.052	6
48 hrs.	82.4	4	0.245	0.073	30
72 hrs.	—	4	0.165	0.118	72
Normal subjects (7)					
24 hrs.	—	—	0.039–0.225	0.000–0.060	
48 hrs.	—	—	0.040–0.188	0.028–0.173	

Accuracy of method in serum determinations: sd 0.003% of dose/liter at 0.050% of dose/liter level.

* Conversion ratio = B/A.

Data of urinalysis (which was carried out in several fractions) and serum analysis during both tests in Case 2 were arranged on a semilogarithmic scale. One group of lines showed serum data of both tests, with the presumption that the PBI^{131} data were *in*correct, and that the *total* serum radioactivity was the correct value for radioiodide. Another group of lines showed the fall of serum radioiodide concentration, as obtained from the difference (total activity $-$ PBI^{131}). Only the latter lines fullfilled the requirement of a constant rate of disappearance of radioiodide from the blood. Moreover the slope was in good agreement with the slope of disappearance of radioiodide from the body, as calculated from the data of fractional urinalysis (Fig. 1).

To explain these results in cases of postoperative myxedema we offer the following hypothesis. The only supposition to be made is that after surgical treatment only very small remnants of thyroid gland tissue are

left functioning, and that these remnants (under continuing, and perhaps increasing TSH stimulation) preserve the typical properties of the total gland in hyperthyroidism. An essential feature of these glands is the rapid turnover-rate of iodine with only slight storage of organically bound iodine. The total iodine content in a hyperfunctioning gland is considerably less than in a normal gland. (In the very small remnants in our cases this "glandular" pool must have been extremely small.) As a consequence, all radioiodide collected by these remnants will be quickly transferred to the blood after being transformed into radiothyroxine.

If we assume that by surgical intervention the clearance of iodide from the blood by the glandular tissue is reduced to 3 ml. of plasma per minute (one tenth of normal and probably $\pm$ one fiftieth of the original clearance before therapy), then the total fraction of the administered dose that will be handled by the gland remnants will be $3/33 = 9$ per cent (renal clearance $= 30$ ml./min.). This fraction of the dose will be transferred in about forty-eight hours completely into the bloodstream as radiothyroxine. Our assumption of a clearance by the thyroid remnants of 3 ml./min. yields a transference in the first instance through the remnants of 9 per cent of the dose. If total iodine excretion (and intake) are unchanged, the hormone production will be $H' = \frac{E}{100/9-1} = 1/10\ E$ (E is the normal iodine intake

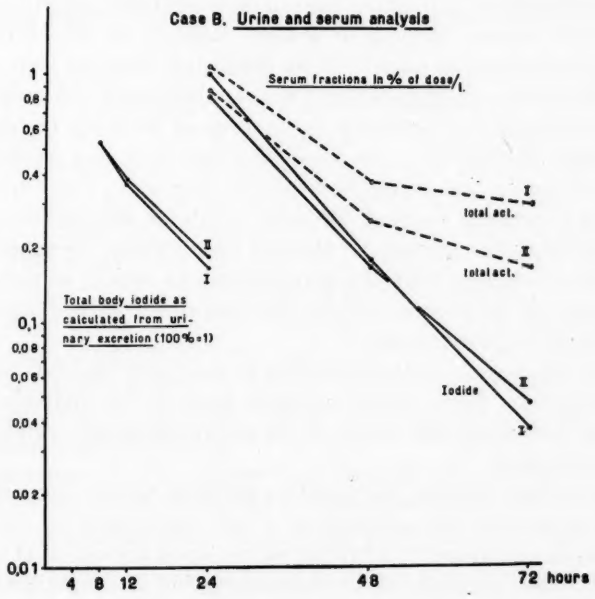

FIGURE 1

and excretion in twenty-four hours). In normal subjects, with an uptake in the gland of 40 per cent of the dose, the hormone production is $H = \frac{E}{100/40-1}$ $= 66/100\ E$ (Stanbury). Thus, with only the small remnants working, hormone production is about one seventh of normal. It is quite conceivable that with this production, myxedema results. As the turnover-rate of the PBI compartment is relatively slow (half-time, ±twelve days), the loss from this compartment in the first few days is negligible.

Estimations of the volume of the extrathyroidal PBI pool (=PBI-space) vary between 25 and 50 liters. Distribution of 9 per cent of the dose in this compartment thus will yield a concentration of PBI^{131} of 9/25 to 9/50 =0.360 to 0.180 per cent of the dose per liter. As the rate of mixing in this compartment will be rather slow, serum values will for this reason tend to be higher, which may compensate for our neglect of the losses in the first few days.

The PBI value of 1.3 microgram per 100 ml. in Case 2 suggests a PBI pool that is about one fifth of normal in weight. Therefore, a more rapid turnover in the PBI compartment than in normal subjects need not be considered (twelve days, according to Riggs).

It is surprising that observations like these reported here, have not been made earlier. Keating (1949) has reported the early formation of PBI^{131} in cases of myxedema (±0.05 per cent of dose per liter), and has suggested extrathyroidal organic binding of iodine. Reports on the formation of PBI^{131} in such important quantities as presented here, we were unable to find in the literature. Riggs came very near to suggesting the train of events that we have assumed. Discussing the findings of Keating, he has written: "A more likely alternative is the possibility that even in a patient with no detectable accumulation of radioactivity in the neck, there may remain small bits of functional thyroid. Because of intense stimulation by thyrotropic hormone such remnants of thyroid tissue would be expected to secrete whatever hormone they can manufacture as rapidly as possible. This would account for the prompt but very limited appearance of organic radioactive iodine in the bloodstream."

We do not agree with his suggestion as to the "very limited" appearance in the bloodstream. However the essential point in our analysis—the lack of a diluting pool of organic iodine in the gland remnants—can be deduced from his conception.

If our hypothesis is right, the question arises as to why cases of myxedema do not show this phenomenon as a rule. According to our analysis, this can only be explained by a further reduction in the size of the remnants to extremely small particles, or by the assumption of a remaining capacity

to pool organic iodine, notwithstanding the intensive stimulation with TSH.

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EVALUATION OF ADRENAL STEROIDS ADMINISTERED INTRAVENOUSLY, INTRAMUSCULARLY AND ORALLY*

TO THE EDITOR:

As part of a study designed to evaluate the relative efficiency of different adrenal steroids and of corticotropin preparations in human subjects, eosinophil counts have been performed at specific intervals, in the course of a 24-hour period during and following the administration of such material.

Cortisone acetate (compound E) was administered intramuscularly and orally. Hydrocortisone (compound F) (free alcohol) was administered intravenously, orally, and intramuscularly. Hydrocortisone acetate was administered orally and intramuscularly. In each instance the dosage was 100 mg., the oral material being given hourly in an amount of 20 mg. for a five-hour period, the intramuscular material in a single amount of 100 mg., and the intravenous material in a volume of 500 cc. of 5 per cent glucose in distilled water infused over a five-hour period.

The results of such studies in 2 patients, one a young woman with atopic dermatitis and the other an adult male with asthma, are shown in Figures 1 and 2. Both patients prior to therapy had high eosinophil counts. In each the eosinophils were relatively "labile" in relation to therapy. The results may be summarized as follows:

1. Intravenous hydrocortisone (free alcohol) appears to have a demonstrable effect within one hour and a major effect within three hours. There is a significant residual effect twenty-four hours after the start of infusion.

* This study has been supported in part by a grant from Merck & Company.

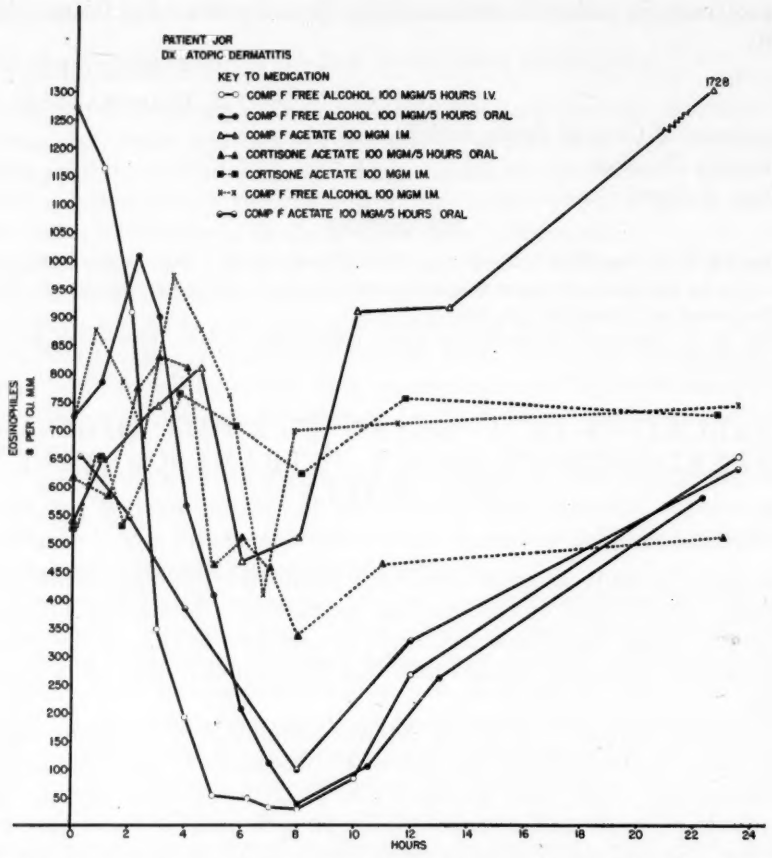
PATIENT JOR
DX ATOPIC DERMATITIS
KEY TO MEDICATION
COMP F FREE ALCOHOL 100 MGM/5 HOURS I.V.
COMP F FREE ALCOHOL 100 MGM/5 HOURS ORAL
COMP F ACETATE 100 MGM I.M.
CORTISONE ACETATE 100 MGM/5 HOURS ORAL
CORTISONE ACETATE 100 MGM I.M.
COMP F FREE ALCOHOL 100 MGM I.M.
COMP F ACETATE 100 MGM/5 HOURS ORAL
1728
EOSINOPHILES # PER CU. MM.
HOURS

FIG. 1. Relative effects of compound E and F preparations on the eosinophils in a patient with severe atopic dermatitis. In each instance, the hours relate to time following initiation of therapy. In the case of intravenous and oral preparations, the eosinophil response accurately mirrors the clinical response. In the case of intramuscular preparations, the long-term clinical response is proportionately greater than the initial eosinophil response.

2. Hydrocortisone free alcohol and acetate, respectively, administered orally produce a major effect within three hours, a maximal effect at about eight hours, and have variable durations of effect.

3. Cortisone acetate administered orally appears to have a similar curve of action, when compared to hydrocortisone acetate and free alcohol, but appears to be moderately less efficient. Additional data are required before one can know what the percentile relationship is.

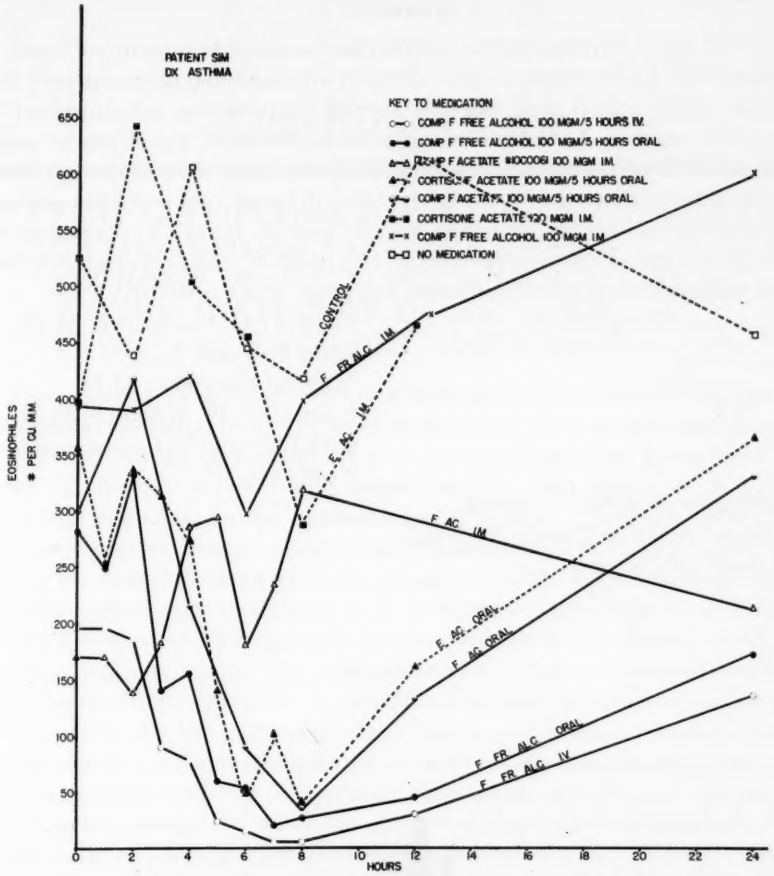
PATIENT SIM
DX ASTHMA
KEY TO MEDICATION
COMP F FREE ALCOHOL 100 MGM/5 HOURS IV.
COMP F FREE ALCOHOL 100 MGM/5 HOURS ORAL
F ACETATE #DC0061 100 MGM I.M.
CORTISONE ACETATE 100 MGM/5 HOURS ORAL
COMP F ACETATE 100 MGM/5 HOURS ORAL
CORTISONE ACETATE 100 MGM I.M.
COMP F FREE ALCOHOL 100 MGM I.M.
NO MEDICATION
EOSINOPHILES # PER CU. M.M.
HOURS
CONTROL
F FR ALC IM
F AC IM
F AC ORAL
F FR ALC ORAL
F FR ALC IV

FIG. 2. Eosinophil response to compound E and F preparations in an asthmatic patient. With the administration of intramuscular preparations, as in the case of the patient with atopic dermatitis, the eosinophil response does not adequately mirror the clinical response.

4. On the basis of this type of assay procedure, intramuscular administration would appear to be an inefficient method for administering either cortisone or hydrocortisone. The total therapeutic efficiency of these preparations administered intramuscularly, however, is considerably less unfavorable than would be indicated by the eosinophil response. Nevertheless it is apparent that the speed of response to all adrenal steroids administered intramuscularly is much slower than is the case when the preparations are given either orally or intravenously.

SUMMARY

On the basis of quantitative evaluation in suitable human subjects, it appears that hydrocortisone (free alcohol) administered intravenously has a more rapid action than adrenal steroid preparations administered in any other manner. At the end of eight hours, however, the effects of cortisone and hydrocortisone administered orally compare favorably with those resulting from intravenous administration, although oral cortisone appears to be somewhat less efficient than hydrocortisone. Intramuscular administration of adrenal steroids, perhaps as the result of slowness of absorption, is an unsatisfactory procedure when a prompt effect is desired.

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HYPERPARATHYROIDISM. CASE REPORT ILLUSTRATING SPONTANEOUS REMISSION DUE TO NECROSIS OF ADENOMA, AND A STUDY OF THE INCIDENCE OF NECROSES IN PARATHYROID ADENOMAS*

The majority of patients suffering with the clinical entity hyperparathyroidism are found to have adenomas of one or more parathyroid glands, and the pathologic physiology of the disease is corrected by surgical removal of the tumor. One would expect the hormonal output of an hypersecreting adenoma to be approximately constant, perhaps increasing gradually with anatomic enlargement of the tumor, but hardly fluctuating widely from week to week or from month to month. Yet soon after hyperparathyroidism was recognized (1, 2, 3), it began to be noted that certain patients seemed to have spontaneous remissions of the disease (4, 5), as judged symptomatically, radiographically and by serum chemical changes. The clinician may, therefore, be justifiably worried when he is confronted by a patient who has had renal calculi containing calcium, hypercalciuria, hypercalcemia and hypophosphatemia, and perhaps even roentgenographically suspicious evidence of hyperparathyroidism, yet who at the time of examination presents none of the chemical abnormalities upon which a diagnosis of hyperparathyroidism might comfortably rest. It has been our custom, and that of some other clinics, to follow such patients at intervals before dismissing a parathyroid adenoma from the diagnostic possibilities; and on occasions one is rewarded a few months later with clear-cut chemical evidence of hyperparathyroidism. But no feasible explanation has offered itself for these rare instances of spontaneous remissions and relapses in this disease. A patient was seen recently whose parathyroid adenoma was the seat of a spontaneous necrosis; and, coincident with this, the hyperparathyroidism disappeared just as if the adenoma had been surgically removed. We were thereby prompted to review the available specimens of parathyroid tumors which had been surgically removed in this hospital for evidence of previous necrosis.

* The studies herein reported were supported in part by a contract between the Atomic Energy Commission and the Johns Hopkins University.

TABLE 1. LABORATORY DATA ON *G.S.* (*J.H.H.* #563600)

Date 1951	*Serum*									*Blood*		*Urine*			Remarks
	Ca mg./100 ml.	P mg./100 ml.	Alk. phospha-tase (Bod. units)	Tot. prot. Gm. %	NPN mg./100 ml.	Na mEq./L.	K mEq./L.	CO_2 mEq./L.	Cl mEq./L.	Hemato-crit %	WBC per cu. mm.	Alb.	PSP	24-hr Ca* (mg.)	
Oct. 6					67	135	3.6	26.6	95.5	32.0	5000				
Oct. 8	12.7	3.5	10.1	7.2	64	130	3.4	25.0	95.0	33.0		1+			
Oct. 13	12.8	3.0		8.0											Transfusion 1000 cc. blood
Oct. 15	14.8	2.7	13.5		52					38.0	5600	1+	12%‡		
Oct. 18														204	
Oct. 19														168	
Oct. 20	20.2	5.3		8.1	77				93.0						Nausea, vomiting, tachycardia (110–120)
Oct. 21														89	Sore throat; increased tenderness of nodule in neck
Oct. 22	13.1	2.3	11.3	6.9	75	129	2.9	27.0	83.5	35.0	10000	1+		111	
Oct. 23	10.7	1.7		7.5										96	
Oct. 24														79	
Oct. 26	8.9	1.8		8.2	60							1+			
Oct. 28										36.0	4400			16	
Oct. 29	8.2	1.6		8.0	47	133	2.7	23.3	90.8	35.0	4900			93	950 mg. Ca i.v.†
Oct. 31														46	
Nov. 1														22	
Nov. 2	9.2	1.4		7.2											Parathyroid adenoma removed
4 hr. post-op.	8.7	1.3		6.7											
Nov. 3	8.3	1.2		6.6										15	
Nov. 5	8.0	1.1		6.5											
Nov. 7	7.9	1.2	18.0	6.4	34	143	4.5	25.0	108.0	27.0	4500				
Nov. 9	8.2	1.4		6.4									11% (1 hr.)		Mild paresthesias. Began 40% $CaCl_2$, 8 cc. t.i.d.
Nov. 10														40	
Nov. 11														31	
Nov. 12	9.0	1.4		6.4						26.5	4200			102	
Nov. 13	9.7	1.4	15.3		29									106	Discontinued $CaCl_2$
Nov. 14														68	
Nov. 15						140	5.1	17.4	108					63	
Nov. 16														32	
Nov. 17	9.3	1.4		7.1						24.0	4500	0		172	880 mg. Ca i.v.†
Nov. 18	9.9	1.3		6.5											Discharged
Dec. 12	10.2				30								29%‡		
Dec. 14										29.0	4500				
1952															
Jan. 5	9.7	2.7													
Feb. 5		2.7	8.5	7.0											
Sept. 12	10.7	3.3		7.1	42										
Nov. 14	10.2	3.4	3.7		32			25.8	107	39.7		0	24%‡		

* Dietary calcium not constant, but always less than 0.5 Gm. daily.
† Given over a 4-hour period as intravenous calcium test.
‡ Two hours.

CASE REPORT

G.S. (J.H.H. #563600), a 57-year-old colored housewife, was admitted to the hospital on October 5, 1951 from the Accident Ward because of epigastric pains and vomiting for the previous week. During the previous year, she had twice appeared in the Outpatient Department with similar complaints and vague joint pains but had failed to return for follow-up studies. The initial impression at both these visits had been peptic ulcer, osteoarthritis and secondary anemia.

Past history: The patient had never been pregnant and menses had ceased uneventfully at age 45. For twenty years, in the spring and fall, she had suffered with postprandial epigastric distress which her physician had diagnosed as "ulcer"; the symptoms had always disappeared within a few weeks on a milk diet supplemented by a dose of soda once or twice weekly. For many years she had been constipated, requiring frequent laxatives. Two years prior to admission the patient had noted nocturia, polyuria and polydypsia, together with episodes of aching in the knees, elbows and shoulders, and of tingling, crampy sensations in the legs nocturnally. There had never been swelling or redness of the joints. The patient told of having taken iron often in the past for anemia. Her appendix had been removed forty-six years previously, and a fatty tumor had been excised from the left breast twenty-six years previously. Nineteen years before entry and again five years before, a fistula in ano had been repaired.

Present illness: Two weeks prior to admission, with return of epigastric pains, intermittent vomiting developed, and for forty-eight hours the patient had vomited even the water drunk. There had never been hematemesis or melena. She had noted generalized itching for the previous week. Her usual weight was 135 pounds, and she thought there had been recent weight loss. Her doctor had prescribed digitalis one week before admission because the patient had complained of shortness of breath.

Examination: Rectal temperature, 99.2° F.; pulse 68, regular; respirations, 20; blood pressure, 110/60; weight, 122 pounds. The patient appeared chronically ill, somewhat undernourished and dehydrated, but alert. She was apprehensive and querulous. The mucous membranes were slightly pale. There was no icterus. Pupils, extraocular movements and fundi were normal. Nose, ears and oral cavity appeared normal. There was no glandular enlargement. There was a small nodule in the area of the right thyroid isthmus but no signs of thyroid dysfunction. By palpation and percussion there was some left ventricular enlargement which was verified by teleoroentgenogram, and there were systolic murmurs present in both the apical and aortic areas. Lungs were clear, with good excursion. There were no abnormal findings in the abdomen. The pelvis was normal on examination. Reflexes and peripheral circulation were normal.

Initial laboratory data: Urine—specific gravity 1.002, alkaline, trace of albumin, no sugar, rare R.B.C., 10–15 W.B.C., no casts. Blood morphology—hemoglobin 9.2 grams, hematocrit 32, W.B.C. 5,000, smear and differential counts normal, sedimentation rate 36 mm. per hour, corrected. Serologic test for syphilis—negative reaction. Serum—nonprotein nitrogen 67 mg. and sugar 84 mg. per 100 ml.; sodium 135 mEq., potassium 3.6 mEq., chloride 95.5 mEq. and CO_2 25 mEq. per liter. (Subsequent chemical determinations are recorded in Table 1.) Stool—normal in appearance; no occult blood. Electrocardiogram—sinus rhythm with second-degree heart block (Wenckebach phenomenon); digitalis effect; QRS complexes normal; ST3, V1 and V3 sagging, with inverted T waves. X-ray examination of the upper intestinal tract disclosed a deformed, scarred duodenum; the presence or absence of activity in the ulcer could not be stated. There was no free hydrochloric acid in the stomach juice. Gastric analysis was repeated one week later, again without histamine, and free HCl (9 units) was found. Bone mar-

row puncture revealed normal findings. Phenolsulphonphthalein excretion, with good urine volume, was 8 per cent in one hour. A concentration test showed a maximum specific gravity of 1.007.

During the subsequent three weeks of observation, it became clear that the patient suffered from renal insufficiency and peptic ulcer; also, to most of us, primary hyperparathyroidism seemed present. The serum calcium level varied between 12.5 and 14.0 mg. per 100 ml. on a diet of 300 mg. of calcium per day (with large doses of aluminum hydroxide, given for the ulcer symptoms); urinary calcium excretion on two successive days was 204 and 168 mg. per twenty-four hours.

X-ray examination of the bones revealed minimal and questionable evidence of rarefaction. There was no evidence of calcification or stones in the renal areas. Upon swallowing barium there was no evidence of compression of the trachea or deviation of the esophagus.

The patient improved gradually, ulcer symptoms were controlled, the blood nonprotein nitrogen level fell and (after two transfusions) the hematocrit and hemoglobin rose to 36 and 13 grams, respectively. However, on October 20 (coincident, in retrospect, with the patient's complaint of soreness in the region of the nodule in the neck) nausea, vomiting and tachycardia supervened, and the serum calcium concentration rose precipitously to 20 mg. per 100 ml., together with a rise in the concentrations of serum phosphorus and nonprotein nitrogen. Following this extraordinary observation, which was checked and verified, the serum calcium level fell equally precipitously over the next three days to 10.7; three days later it was 8.9 and six days later it was 8.2 mg. per 100 ml., with the serum phosphorus level falling coincidently to 1.5 mg. per 100 ml., at which levels both remained until operation on November 2 (Chart 1). On the basis of these observations, those of us who had been convinced of the diagnosis of hyperparathyroidism were left with no alternative but to believe that the adenoma had spontaneously ceased to function, and so a diagnosis of infarction of the adenoma was made.

At the operation performed by Dr. Thomas Johns, the tumor was found in the region of the right inferior pole of the thyroid. Its size was $3.5 \times 2 \times 2$ cm., and the weight was 6 grams. On section, all but the rim appeared red and slightly softened. There was a pale yellow zone demarcating this central red area from the surrounding pale tan, more firm tissue.

Microscopic sections (S.P. 51-5550) (Figs. 1–4) of the tumor showed a mixture of viable tumor cells arranged in masses or sheets and bounded by varying amounts of fairly cellular connective-tissue septa in which there were macrophages containing hemosiderin pigment. In addition, there were large areas of necrotic tissue, obviously formerly tumor cells. One could

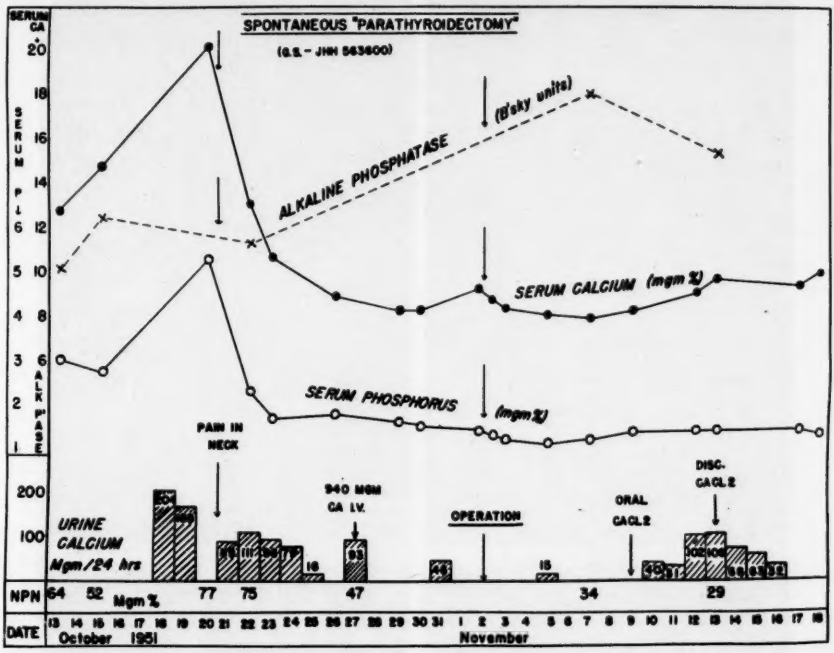

CHART 1. Pertinent data during hospital course of G.S. (#563600).

find a sharp demarcation between these two types of tissue. No normal parathyroid cells were found. Numerous blood vessels, both arteries and veins, had thickened walls but were embedded in the connective tissue septa. No evidence of any fresh vascular occlusion was found.

Our interpretation of the changes which were present was that a large portion of the tumor had recently become necrotic. The reason for this was not clear. In addition there were large areas of connective tissue with hemosiderin-containing macrophages, which would indicate previous areas of now organized necrosis, which would not have been associated with the current episode.

A small specimen of sternum removed at operation showed definite, though not marked, osteitis fibrosa.

In view of this picture the other parathyroid tumors in the files of the Surgical Pathology Laboratory were reviewed. In 3 of the 35 cases available for study, large scars with macrophages containing hemosiderin were found (Figs. 5 and 6). There was no evidence in the clinical record to indicate that in these cases there had been any episode similar to that occurring in the

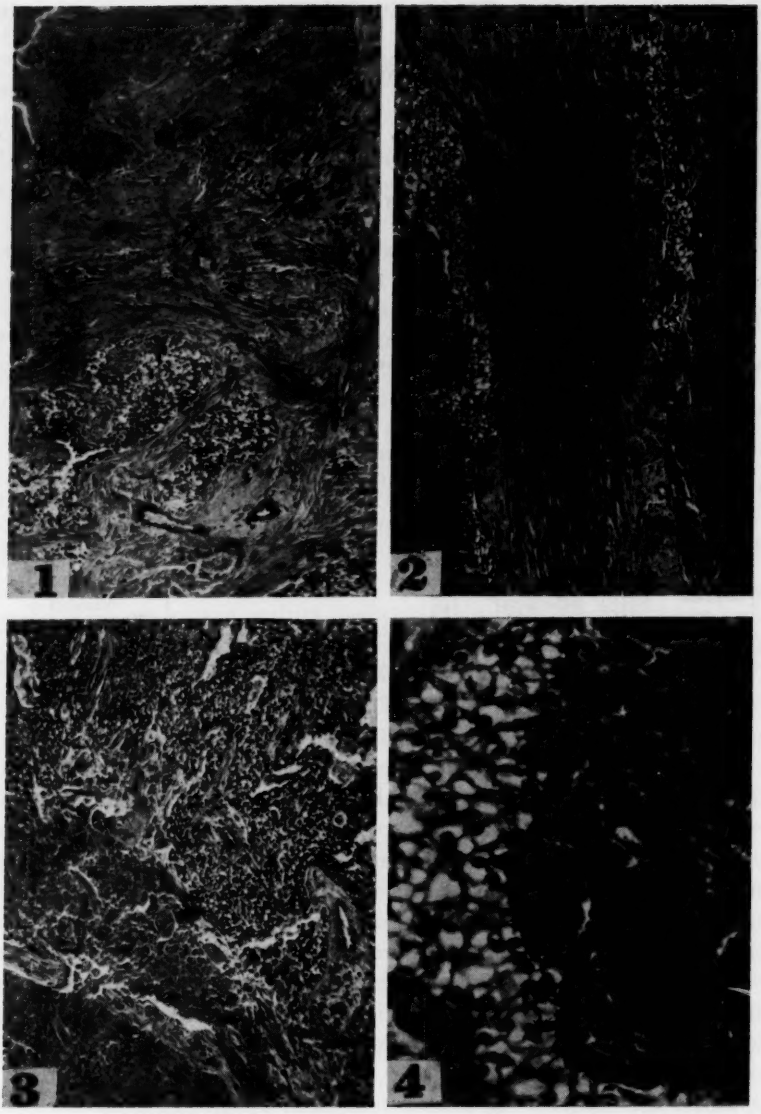

FIG. 1. Low-power microphotograph of tumor (S.P. 51-5550) removed from Patient G.S. Upper field shows necrotic cells; middle portion shows fibrous tissue, beneath which there are nests of tumor cells.

FIG. 2. Another field of same tumor (S.P. 51-5550). In the center is old connective tissue lined by a border of tumor cells and at each side, necrotic cells.

FIG. 3. Another field of tumor (S.P. 51-5550). Necrotic cells are seen in left low portion.

FIG. 4. Higher power of tumor (S.P. 51-5550) to show sharp transition between normal tumor cells and necrotic ones.

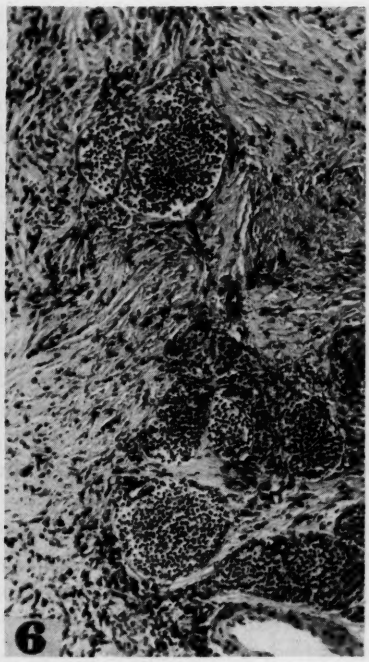

FIG. 5. Tumor (S.P. 94208) to show large scar with dark-staining macrophages containing hemosiderin. Tumor cells are seen in left lower portion.

FIG. 6. Tumor (S.P. 50-4893) to show nests of tumor cells imbedded in fibrous connective tissue. Macrophages containing hemosiderin are present in this stroma.

patient herein described. In 3 other tumors small insignificant scars were found.

The patient's postoperative course was smooth, and convalescence was rapid. Only brief mild paresthesias were noted on the seventh postoperative day, quickly corrected by oral calcium administration. She was dischcarged from the hospital on the sixteenth postoperative day.

The patient was seen in the Out-Patient Department on November 10, 1952, approximately one year after the operation; and excerpts of interest from the note of that date are as follows:

1. Weight—138½ pounds. This is a gain of 32 pounds since operation, the weight having fallen from 122 pounds on admission to 106 pounds at the time of operation.

2. General change—amazing. The patient looks from five to ten years younger. She states she feels better now than in the previous ten to fourteen years. She is well and has no complaints. She plans to get a job.

3. Mental state—greatly changed from a whining, uncooperative person to a bright, smiling, cheerful, cooperative, healthy adult female. Patient states she is less tense and irritable, and her husband has noted that she is now much easier to get along with.

4. Appetite—improved shortly after discharge from the hospital. She eats almost anything since operation with no gastrointestinal distress, and she has had no milk since leaving this hospital. She did have a flare-up of ulcer symptoms ten months postoperatively, consisting of postprandial epigastric gnawing and burning, which cleared completely with aluminum hydroxide and phenobarbital therapy over a two-week period. A gastrointestinal x-ray series in September, 1952 showed duodenal deformity which had not changed from a previous series in October 1951.

Gastric analysis was repeated on September 12, 1952, when the patient was having ulcer symptoms. Gastric contents after alcohol (without histamine) disclosed 42 units of free HCl.

5. Bowel habits—constipated for twenty years prior to operation, requiring milk of magnesia or epsom salts once or twice weekly. However, since operation bowels have been regular and she has taken no laxatives.

6. Joint pains—were especially bad in the knees preoperatively and for a few years before operation she was unable to kneel down to scrub floors. By April 1952 (four to five months postoperatively) these symptoms had disappeared, and she now kneels down to scrub floors once weekly with no difficulty.

7. Polyuria and nocturia—which were major preoperative complaints, disappeared within four or five weeks postoperatively.

DISCUSSION

There have been reported other cases of parathyroid tumors with hemorrhage or infarction. In one instance (6) the patient's first recognized manifestation of abnormal parathyroid function was tetany, which evidently resulted from the abrupt cessation of the tumor's function as a result of "infarction." In other reports (7) there are not enough data available to determine whether the necrotized tumor was functioning. In those cases of our series in which the removed functioning adenoma showed evidence of old necrosis, the histories are inadequate to determine whether or not a temporary physiologic remission accompanied the tissue destruction. It seems altogether possible that such was the case in 3 of these patients in whom the area of tumor necrosis was extensive, as indicated by scars and hemosiderin deposition; and such incidences of necrosis within parathyroid adenomas may explain the observed clinical remissions of the disease (4, 5). Other endocrine tumors appear to have a predilection for spontaneous

remissions and also are not infrequently found at operation or autopsy to be the sites of hemorrhage—particularly pheochromocytomas (8, 9) and pituitary tumors (10, 11, 12).

There are other points of interest in the case history presented here. As judged by the abrupt and transitory rise in serum calcium from mildly hypercalcemic to marked hypercalcemic levels, there must have been massive discharge of parathyroid hormone from the tumor as a result of the acute necrosis. This supposition is fortified by the coincident rise in serum phosphorus concentration to a state of hyperphosphatemia and by the rise in the blood nonprotein nitrogen level—all characteristic phenomena of acute parathyroid intoxication (13, 14, 15). It has recently been pointed out that levels of hypercalcemia which are associated with parathyroid intoxication (20 mg. per 100 ml. or more) are also accompanied by changes in the ultrafilterability of the calcium and phosphorus of the serum (16), and that the clinical manifestations may be the direct result of a hypothetical colloidal calcium-phosphorus protein complex which is formed at such hypercalcemic levels.

It will also be noted that, as is so often the case, the serum phosphorus level remained low for a long time after the parathyroid adenoma had been removed, even though the serum calcium promptly returned to normal or even hypocalcemic concentration. It has been assumed that calcium and phosphorus are being laid down rapidly in new bone once the hyperparathyroid state has been relieved; but, though such an explanation may suffice for a postoperative hypocalcemia (rarely seen in the absence of severe skeletal disease and an elevated level of serum alkaline phosphatase (17)), it will hardly serve to explain the prolonged (sometimes many months) hypophosphatemia which is seen after removal of a high percentage of parathyroid adenomas, even in the absence of demonstrable bone disease and without any hypocalcemia (17). The parathyroid hormone affects renal excretion of phosphorus certainly, but also seems to affect cellular organic-inorganic phosphorus relationships, and these latter effects appear to persist for some time after the other manifestations of a previously existing hyperparathyroidism have disappeared (18).

Of all the pathologic changes accompanying hyperparathyroidism, those in the kidney probably carry the most serious prognosis in regard to long-term survival. Renal damage may result from hyperparathyroidism per se and is not necessarily secondary to renal calculi with consequent obstruction and infection (19). Such was the case in the patient presented here, in whom there was albuminuria and diminution in concentrating power and phenolsulphonphthalein excretion in the absence of radiologically demonstrable renal calcification or calculi. Even though the hyperpara-

thyroidism be corrected surgically, many of these people ultimately go on to die of renal disease (20). However, that all such damage is not necessarily irreversible (21) is well demonstrated in the present patient, in whom there was disappearance of albuminuria and return of serum nonprotein nitrogen to normal levels, following cure of the disease. There was also a considerable improvement in phenolsulphonphthalein excretion within the first two months following operation.

The patient of this report suffered also from a duodenal ulcer. Peptic ulcer is seen in a surprisingly large number of patients with hyperparathyroidism (15 per cent in the Johns Hopkins group (20)) and several have been reported from the Mayo Clinic (22a, b, c). No ready explanation for the coexistence of these two disease states has as yet been offered. In our patient, during active hyperparathyroidism, fasting gastric contents contained but a trace of free hydrochloric acid on two occasions, whereas after the hyperparathyroidism had been corrected, hyperchlorhydria was present during a relapse of ulcer symptoms. Though our patient had drunk large quantities of cow's milk during the brief relapses of her ulcer each spring and fall for twenty years, she had not taken any appreciable quantities of alkali and for the rest of the year did not drink milk because she did not like it.

The effects of abnormal concentrations of serum calcium upon the psyche are demonstrated in this patient's course. The psychoses of hypocalcemia (23) are the more dramatic and frightening, but hyperparathyroidism may also be accompanied by vague nervous and emotional symptoms leading to the diagnosis of psychoneurosis and sometimes even to psychosis (24). Often patients say, after hyperparathyroidism has been corrected, that disposition, sleep and work tolerance have improved tremendously, even though they were scarcely aware of these indispositions before operation.

SUMMARY AND CONCLUSIONS

There is reported the case history of a 57-year-old colored woman who suffered with hyperparathyroidism due to a parathyroid adenoma. A spontaneous acute necrosis within the tumor was accompanied by an immediate brief exacerbation of the disease to a state of acute parathyroid poisoning; then there was a rapid fall in serum calcium to normocalcemic levels and continued hypophosphatemia, just as occurs after surgical removal of a parathyroid adenoma.

Renal function, barely competent before correction of the hyperparathyroidism, improved greatly following operation.

A pathologic review of the other parathyroid adenomas removed in the Johns Hopkins Hospital disclosed evidence of old necrosis and hemorrhage

in 3 of 35 cases. Spontaneous necroses of such tumors is offered as an explanation for spontaneous remissions which are occasionally seen in this disease.

The coincident finding of peptic ulcer and hyperparathyroidism in the same patient and the psychiatric manifestations accompanying hyperparathyroidism are also discussed.

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Editorial

THE DETERMINATION OF URINARY 17-KETOSTEROIDS

THE determination of urinary 17-ketosteroids has become accepted as a valuable aid in the diagnosis of certain endocrine disorders, particularly disorders of the adrenal cortex. Since the methods first proposed by Zimmermann (1, 2), Wu and Chou (3) and Callow, Callow and Emmens (4) were reported, they have undergone modification at the hands of many investigators. In view of the continued and increasing interest in this determination, it seems pertinent to consider the adequacy of some of the proposed methods. This discussion will be limited to the colorimetric reaction, often called the Zimmermann reaction when applied to steroids.

Callow and associates used purified absolute alcohol as the solvent for the test substance and the two reagents (KOH and *m*-dinitrobenzene) involved in this reaction. In most of the modifications aqueous alkali has been substituted for the absolute alcoholic alkali, since the latter solution is subject to rather rapid deterioration unless certain precautions are taken. Many who use aqueous alkali apparently are not aware of, or ignore the fact that its use may lead to gross errors. In urinary extracts there are nonketonic chromogens for which the usual blank is not adequate compensation. Furthermore it has been shown repeatedly that the color developed in urinary extracts in the presence of more than traces of water does not, in general, follow Beer's law; that is, the intensity of the color is not proportional to the amount of urinary extract. It has been emphasized recently that a 17-ketosteroid added to urinary extracts does not yield as much additional color as would be expected from the amount of pure steroid used and it has been proposed to add known amounts of a 17-ketosteroid to urinary extracts as internal standards. This situation would be expected from the knowledge that deviations from Beer's law usually occur. These deviations vary from urine to urine.

It would be presumptuous to state that any particular method gives erroneous results, unless one had made a thorough study of the method. Parenthetically, most of the proposed methods have not received adequate study by their proponents. Nevertheless, one gains the impression that those methods which involve the use of aqueous alkali may be particularly inaccurate when the 17-ketosteroid level is very low, as in Addison's disease. In these cases the ratio of nonketonic chromogens to 17-ketosteroids is high and very often it is impossible to determine definitely whether or not any 17-ketosteroids are present, even with the best available colorimetric procedure.

In the case of very young female pseudohermaphrodites the 17-ketosteroid levels may be relatively high for children of that age group but the absolute amounts may be only a few milligrams. When such patients are treated with cortisone, the dose may be so regulated that the 17-ketosteroid excretion is brought to a normal level. When the decrease in 17-ketosteroid excretion is only a few milligrams, an accurate method would seem to be essential for adequate control of the dose of cortisone.

It is often stated that a method is "good enough for clinical purposes." Unfortunately the attitude expressed by this statement is extended to tolerate grossly inaccurate methods. A much better case can be made for the use of the very best method which it is practical to use for clinical purposes. The Committee on Clinical Endocrinology of the British Medical Research Council (5) recently has recommended a procedure for the determination of the urinary 17-ketosteroids which it is believed fulfills this description. The Committee recommended the method of Robbie and Gibson (6) for hydrolysis and extraction, the method of Callow and associates (4) for colorimetry and the formula of Talbot and associates (7) for correction of the result. It would be of value to all those interested in this subject if some such standard method could be adopted in this country. Actually, the preparation and use of absolute alcoholic potassium hydroxide, as in the method of Callow and associates (4) is not as much of a chore as is generally assumed. It would be well if the use of aqueous alkali were abandoned.

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Questions and Answers

(Questions submitted at the 1952 Postgraduate Assembly of the Endocrine Society)

Answers by Peter H. Forsham, San Francisco, California

QUESTION: Is there any specific means of suppressing or antagonizing desoxycorticosterone?

ANSWER: The output by the human adrenal of desoxycorticosterone-like hormones may be suppressed by the administration of small amounts of cortisone acetate (25 to 37½ mg. a day), which will inhibit endogenous ACTH production and thereby diminish the output of desoxycorticosterone-like hormones together with all the other adrenal cortical steroids. This has been demonstrated conclusively, however, only in patients with the adrenogenital syndrome and hypertension, by Wilkins and his associates. In the average hypertensive patient there is usually but little depression of blood pressure when using cortisone as an ACTH suppressant. As to antagonizing the action of desoxycorticosterone, cortisone and hydrocortisone will do this all along the line. Cerebral excitability is increased by the cortisones, and decreased by desoxycorticosterone. Hydration of the red blood corpuscles and other cells following ingestion of large amounts of water, is increased by desoxycorticosterone and decreased by the cortisones. When under the action of desoxycorticosterone, an excessive amount of sodium is retained by the renal tubules; a relatively smaller amount of cortisone administered in addition to the desoxycorticosterone will produce a sodium diuresis, presumably by replacing the highly active salt retainer (DCA) by one that is only one-fiftieth as active (compound E or F). These points illustrate the major features of the cortisone-desoxycorticosterone antagonism, of which there are numerous other examples.

QUESTION: How much DCA is found normally in the body?

ANSWER: Desoxycorticosterone acetate (DCA) has not been found in man. It has been isolated from hog adrenals in very small amounts only and it was questionable whether it did not represent an artifact. It has, however, been shown by chromatography in the effluent of a perfused ox adrenal, by Pincus and his associates. It may be stated that if present at all in man, there is too little to demonstrate in blood and urine. On the other hand, a more highly polar steroid which has some twenty to thirty times the sodium-retaining potency of desoxycorticosterone acetate has been demonstrated chromatographically in the blood and urine of human subjects; this has been done independently in London and San Francisco by Simpson *et al.* and by Luetscher *et al.*, respectively. One may anticipate some interesting developments as this material is further characterized and obtained in quantity large enough to test all of its physiologic functions.

QUESTION: Please elaborate on the two-year investigation with ACTH, based on findings that are in error because of poor absorption or destruction when given intramuscularly.

ANSWER: The vast majority of commercial ACTH preparations undergo inactivation when given intramuscularly. This inactivation ranges from 90 to 30 per cent. Some of the preparations on the market between 1948 and 1950 underwent severe inactivation when administered intramuscularly. Most present-day preparations are inactivated minimally. The effects (in terms of USP units) are now relatively greater with more stable preparations. When using corticotropin gels expressed as house units, the dosages are identical with those found in the literature of the years 1947–1950—being prepared that way by the manufacturers.

QUESTION: Please comment on technical difficulties and methods of determining mineralocorticoids.

ANSWER: Mineralocorticoids may be characterized by their ability to effect sodium retention and transitory potassium loss in the kidney, the sweat glands and the gastrointestinal tract. Methods which utilize this characteristic are never specific, since glucocorticoids also have a moderate sodium-retaining activity. The best method consists of using an adrenalectomized rat and measuring the urinary Na-K ratio before and following the injection of a biologic extract containing mineralocorticoids. As with any bioassay, the wide variations require a large number of animals. Other methods involve the determination of radioactive Na and K, a procedure which facilitates the technique. By the use of chromatography some of the glucocorticoids can be eliminated. A concentrate of steroids more nearly resembling mineralocorticoids, with twenty to thirty times the activity of desoxycorticosterone acetate, has been obtained in a venous adrenal effluate (Simpson and Tait) and in human urine (Leutscher).

The fibroblastic activity of mineralocorticoids may be contrasted experimentally with the antifibroblastic effect of certain glucocorticoids; quantitative assay methods are thus made available. Convulsive thresholds of the central nervous system to faradic stimulation may be used similarly.

QUESTION: What is the mode of action of ACTH and cortisone in acute inflammatory disease or acute collagen disease, *e.g.*, peritonitis, typhoid, etc.?

ANSWER: ACTH and cortisone change the cellular reaction to foreign bodies, both physical and bacterial. There is less leakage across the endothelium of capillaries and thus a decrease in the local swelling. There are fewer neutrophils arriving on the scene and more active phagocytes. Above all, there is practically no fibroplasia, so that the walling-off process is poor. However, the inhibition of the spreading factor (hyaluronidase) by these agents is a redeeming feature. The lack of localization which ensues is unfavorable, but it is also favorable because it allows antibodies and antibiotics to penetrate to the site of infection. Since the cortisones abolish all the systemic signs of infection such as fever and malaise, the patient as a whole will do better, eat more, and the blood pressure will be more stable. The use of ACTH and cortisone in infectious diseases is highly controversial at this time and fraught with the danger of the spread of infectious organisms. However, when effective antibiotics are available and the causative organisms are susceptible, ACTH or cortisone may tip the scale in favor of the survival of a patient who might otherwise die of overwhelming sepsis and shock.

QUESTION: Would you recommend as a routine procedure the Thorn test in surgical cases with arterial hypotension?

ANSWER: Yes. If the eosinophils fall less than 50 per cent after administering 25 USP

units of ACTH intramuscularly over a four-hour period one should either follow up with an eight-hour intravenous infusion of the same amount, counting eosinophils at zero and eight hours, or assume that the result of the test is correct and treat the patient prophylactically for adrenal insufficiency. The administration of 200 mg. of cortisone acetate intramuscularly the night before and on the morning of surgery, and 50 mg. every six hours intramuscularly for the first twenty-four hours will prevent adrenal insufficiency during major surgery, even if the patient suffers from Addison's disease.

QUESTION: Will ACTH lower the eosinophil count in patients with intestinal parasites?

ANSWER: In nearly all instances of intestinal parasitic infestation ACTH will lower the eosinophil count by the usual 50–70 per cent in four hours following 25 USP units administered intramuscularly. The percentile fall will, however, be diminished on occasions when a very acute stage of the infestation is present. This extremely rare coincidence is due to the excessive production of eosinophils by the bone marrow at a rate greater than the rate of destruction initiated by ACTH or cortisone.

QUESTION: Have you had any experience with the use of cortisone in keloid scars?

ANSWER: No. One may predict, however, that the use of cortisone in the prevention of keloid scars can only be partly successful, since the prevention of fibroplasia (which will inhibit keloid formation) will also prevent adequate wound healing, and therefore a partial compromise is all that can be hoped for. It may be worth a try in selected cases.

QUESTION: Hirsutism (mild or moderate) not infrequently develops in postmenopausal women. This may well be adrenal in origin. If so, how does this measure up with the statement that corticotropins decrease with ascending FSH values in the menopause?

ANSWER: It is likely that the hirsutism of postmenopausal women is connected with adrenal androgenic overactivity but this has not been proved. It is equally possible that the hilar cell rests of the ovaries, which secrete testosterone-like substances, become overactive as a result of the involution of the remainder of the ovary. If one accepts this latter hypothesis, a temporary decrease in corticotropin early in the menopause should not prove incompatible with an increase in hirsutism at that time.

QUESTION: How do you explain lack of the usual clinical response in a person with perforation of an ulcer occurring during cortisone therapy?

ANSWER: Since cortisone over prolonged periods of time and in doses of more than 50 mg. a day will tend to reduce fibroplasia, ulcers might tend to perforate. Since the inflammatory response is changed under the action of this drug in such a way as to diminish the walling-off process, peritonitis may develop. In the absence of the usual inflammatory responses there will be no marked signs of this process and it may be diagnosed only by noting the most minute changes with great care.

QUESTION: Would you advise cortisone in the emergency treatment of snake bite? In addition to antivenom?

ANSWER: Cortisone or ACTH will add to the treatment of snake bite by reducing the effect of antigen-antibody complexes, which account for some of the ill effects of the bite. In addition to antivenom, one should administer 200 mg. of cortisone acetate by mouth

and follow this by 50 mg. every six hours for three more doses, or else give 80 units of corticotropin gel and give 40 units, eight and then again twelve hours later.

Answers by Ira T. Nathanson, Boston, Mass.

QUESTION: Do you uniformly perform ovarian ablation in premenopausal women who undergo a radical mastectomy?

ANSWER: Although there is still disagreement concerning the role of castration in the management of breast cancer, and the optimum time for its performance, a practical working plan has emerged. The beneficial effects are essentially confined to premenopausal women, though occasionally castration appears to be of value a few years after spontaneous cessation of the menses, if there is evidence of residual ovarian activity. In the male, by contrast, orchiectomy seems to be more efficacious in older individuals with advanced breast cancer.

Castration is primarily of value as a palliative procedure. Present data do not indicate that it is significantly advantageous as a prophylactic measure in conjunction with radical mastectomy if the latter is performed with the expectancy of control of the disease. Beneficial effects of castration, whenever instituted, are of limited duration. Thus, the trend is to withhold the procedure for treatment of manifestations beyond effective control by the usual methods. In this way, further benefit may be obtained and the life expectancy of the patient may possibly be prolonged.

QUESTION: Please comment on the treatment of painful puberal breast, hypertrophy and mastodynia in general.

ANSWER: A. The pain in the puberal breast usually subsides spontaneously. In the female, pain frequently subsides as development continues and in the male, as the usually transient mammoplasia undergoes regression. Persistence of pain in the male may be treated by androgens, but this should be reserved only for exceptionally prolonged symptoms. Testosterone in relatively low dosages for short periods, either by oral or peroral administration, is often sufficient. It is unwise to prolong this therapy, since it may upset the delicate hormonal balance at puberty. Surgical removal of the breast tissue in the male should be considered primarily for psychologic reasons in the exceptional case in which pain and hypertrophy persist.

B. There is no effective treatment for the hypertrophic breast in the female, except surgery. This should be reserved for the very exceptional case.

C. Mastodynia in the female may be treated as follows: When the symptom is primarily excessive weight or a pendulous breast with a narrow attachment to the chest wall, an uplift support is usually sufficient to obtain relief. Mastodynia is frequently caused by edema and increased vascularity in the premenstruum. In this instance, support combined with the administration of ammonium chloride at dosage levels of 4 to 6 grams daily for two weeks preceding the anticipated menses may alleviate the symptoms. Testosterone may be of benefit if the foregoing measures fail, but it should generally be used in conjunction with them. This hormone is usually administered two weeks or less before the expected menses. The total dosage per month should not exceed 300 mg. or be prolonged for intervals of more than three months, in order to avoid virilizing effects in some patients. Often symptoms are relieved during this time interval. With recurrence of symptoms the regimen may be repeated. Rest periods are desirable under any circumstance.

The Endocrine Society

THE SIXTH ANNUAL POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

SPONSORED JOINTLY BY THE ENDOCRINE SOCIETY AND THE UNIVERSITY OF MINNESOTA AT THE CENTER FOR CONTINUATION STUDY OF THE UNIVERSITY OF MINNESOTA MEDICAL SCHOOL, MINNEAPOLIS, MINNESOTA

September 28–October 3, 1953

The faculty will consist of fifteen clinicians and investigators eminently qualified in the fields of endocrinology and metabolism. The program will consist of lectures, demonstrations of laboratory tests, presentation of cases, and question and answer panel discussions.

The forty-five lectures to be presented will cover most of the phases of endocrinology and certain metabolic diseases. The course is designed to give a broad basis of understanding of diagnosis and therapy in the field of endocrinology and metabolism for the man in general practice and for those in other specialties who wish to have a general knowledge of this rapidly growing field.

A syllabus with brief abstracts of the lectures will be available to the registrants at the time of the Assembly.

For further information concerning the program, write to the Director, Center for Continuation Study, University of Minnesota, Minneapolis 14, Minnesota. Arrangements for housing accommodations will be made through the Center for Continuation Study. Tuition fee, $100.00.

(For *program*, see the July issue of the Journal.)

THE 1954 ANNUAL MEETING

The next Annual Meeting of the Endocrine Society will be held in the Sir Francis Drake Hotel, San Francisco, California, June 17, 18 and 19, 1954.

RECIPIENTS OF AWARDS FOR 1953

CIBA AWARD

The recipients of the Ciba Award for 1953 are *Sidney Roberts*, Associate Professor of Physiological Chemistry at the School of Medicine, University of California at Los Angeles and his wife *Clara Marion Szego*, Assistant Professor of Zoology at this University.

Dr. and Mrs. Roberts both received their doctor's degrees in Physiological Chemistry at the University of Minnesota, Mrs. Roberts in 1942, preceding her husband by one year in the achievement of this degree. It is also presumed, although not verified, that Dr. Samuel's laboratory not only furnished Dr. Roberts with the facilities for advanced study but also the opportunity to persuade the then Miss Szego that collaboration is of value not only in research but in other areas of human activity. Nevertheless, whatever may have been the circumstances that led to their meeting, it must now be recorded that these young people in the course of the last ten years have made significant contributions to endocrinology. It should also be known at this time that your Awards Committee found it so impossible to decide whether husband or wife deserved recognition that they decided to make a joint award, the first time this has been done in the case of this award of the Society.

The contributions made by Dr. and/or Mrs. Roberts fall into three main groups. Their first studies, in collaboration with Drs. Samuels and Reinecke, demonstrated that the previous dietary habits of animals not only greatly modified the capacity of the tissues to utilize the various foodstuffs, but also the response to such hormones as insulin. It was also shown that the quantity as well as the composition of the diet determined the capacity of hypophysectomized rats to endure fasting, being greatest when adequate calories and a diet of high fat content were given. These observations emphasize the fact that the nutritive state of the tissues is a major factor in determining their responsiveness to hormones.

Dr. and Mrs. Roberts then began their well known studies on the form in which estrogens are transported in the blood, and the role played by the liver in the activity of these hormones on the reproductive tract. They were able to demonstrate that practically all the circulating estrogen is combined with a lipo-protein from which it is readily separated by simple diffusion. Since the comparative insolubility of the steroids in water has made it difficult to account for their transport in the blood, the knowledge that they are carried as a lipo-protein complex, which readily yields the steroid at cell surfaces, is a matter of no little importance.

More recently, Dr. Roberts has been engaged in a study of the sites of origin of the serum proteins and the influence on their production of the adrenal cortical and other hormones. He has been able to show that the release of protein by liver and spleen slices is enhanced by previous treatment of the animal with cortical hormone and is depressed after adrenalectomy. These studies promise to throw light on the well known effect of the adrenal cortical hormones on protein metabolism.

In making the Ciba award for 1953 to Dr. and Mrs. Roberts, the Endocrine Society congratulates them on their present achievements and wishes them well in their future work.

SQUIBB AWARD

This year The Endocrine Society is particularly proud to bestow its highest honor, the Squibb Award, upon a great man whose pioneering research, begun nearly a half century ago, has stood the test of time. Our modern knowledge of the physiology and chemistry of the thyroid gland stems largely from work begun in 1905 by a young man named *David Marine.*

David Marine was born in 1880. After obtaining a B.S. degree from Western Maryland College, he graduated from the Johns Hopkins University School of Medicine in 1905. He then went to Cleveland to accept a position in the department of pathology at Western Reserve. Immediately his scientific curiosity was aroused by the high incidence of goiters which he observed in both dogs and human beings. There followed a series of physiological, chemical and histological studies which were carefully planned and painstakingly executed over the next fifteen or twenty years. These showed that the large goitrous thyroids were deficient in hormonal activity and that this deficiency was correlated with absence of iodine. Marine concluded that iodine must be an essential constituent of thyroid hormone. The idea of this young man was ridiculed by the great Swiss authorities Kocher and Wegelin. Had not Tschirch in Berne shown that no iodine was present in the sheep's thyroid? It is true that Baumann in Germany had reported the presence of iodine in 1896, but could this substance be of any real importance if, as Marine claimed, the daily requirement was only 1 milligram? Marine continued patiently with one experiment after another. Finally, in the face of considerable opposition, he was able to make crucial large-scale human experiments demonstrating the prophylactic value of iodine and making possible the control of endemic goiter throughout the world. Marine's descriptions of hyperplasia and involution of the thyroid gland are classics. Perhaps less well known is the fact that he suggested the use of Lugol's solution in the preoperative preparation of patients with Graves' disease, an idea rejected by Crile but adopted twelve years later by Plummer.

Dr. Marine's contributions to endocrinology, made at Western Reserve from 1905 to 1920 and as Director of the Laboratory Division of the Montefiore Hospital in New York between 1920 and 1945, were not limited to the study of the thyroid gland nor were the subjects and methods antiquated, as shown by the titles and dates of such papers as: "Hypertrophy and Hyperplasia of Parathyroids in Birds"—1912; "Tetany in Dogs"—1914; "Relation of Suprarenal Cortex to Thyroid and Thymus"—1926; "Suprarenalectomy and Injection of Sodium Salts"—1927; "Cyanides and Goiter"—1932; "Periarteritis nodosa-like Lesions in Thiouracil-fed Rats"—1945.

We trust that the bestowal of the 1953 Squibb Award will emphasize to a younger generation of scientists that the introduction of isotopic methods and new techniques has served merely to confirm the sturdiness of the concepts of David Marine. The Awards Committee wishes to thank this modest man for allowing us to drag him from his peaceful retirement by the sea at Rehobeth Beach that we may look this night upon one of the great pioneers of endocrinology.

SCHERING FELLOWSHIP

The Schering Fellowship in Endocrinology for 1953 is awarded to *Mr. James E. Ashmore.*

Mr. Ashmore received the Bachelor of Science degree in 1947 and the Master of Science degree in chemistry in 1948 from the North Texas State College, where he worked on the synthesis of steroids under Dr. Max Huffman at the Southwestern Medical College. This year he will receive the degree of Doctor of Philosophy in Biochemistry from St. Louis University, where he has been working with Dr. E. A. Doisy on the excretion of the metabolites of radiotestosterone in the rat.

The Fellowship is awarded to him so that he may broaden his experience with special training in the physiological aspects of endocrine research. He plans to investigate, under Dr. A. Baird Hastings of Harvard University, the effects of adrenal cortical hormones on inorganic and carbohydrate metabolism in the rat liver and diaphragm.

The American Goiter Association

The next Annual Meeting of the American Goiter Association will be held in the Hotel Somerset, Boston, Massachusetts, April 29, 30 and May 1, 1954.

VAN METER PRIZE AWARD FOR 1954

The American Goiter Association again offers the Van Meter Prize Award of Three Hundred Dollars and two honorable mentions for the best essays submitted concerning original work on problems related to the thyroid gland. The award will be made at the annual meeting of the Association which will be held in Boston, Massachusetts, April 29, 30 and May 1, 1954, providing essays of sufficient merit are presented in competition.

The competing essays may cover either clinical or research investigations; should not exceed three thousand words in length; must be presented in English, and a typewritten double-spaced copy in duplicate sent to the Corresponding Secretary, Dr. John C. McClintock, 149½ Washington Avenue, Albany 10, New York, not later than *January 15, 1954*. The committee, who will review the manuscripts, is composed of men well qualified to judge the merits of the competing essays.

A place will be reserved on the program of the annual meeting for the presentation of the Prize Award Essay by the author, if it is possible for him to attend. The essay will be published in the annual Transactions of the Association.

BOOKS RECEIVED

American Lecture Series. Charles C Thomas, Publisher, Springfield, Illinois.

Contact Dermatitis. By GEORGE L. WALDBOTT, M.D., Senior Physician, Harper Hospital, Detroit, Michigan. A monograph in the Bannerstone Division of American Lectures in Dermatology, edited by Arthur C. Curtis, M.D.; 232 pages; 332 illustrations; 1953.

The Diabetic Neuropathies. By JOSEPH I. GOODMAN, M.D., Senior Clinical Instructor, Western Reserve University, Senior Visiting Physician and Physician in Charge of Diabetic Clinic, Mount Sinai Hospital, Cleveland, Ohio, SIEGFRIED BAUMOEL, M.D., LEONARD FRANKEL, M.D., LOUIS J. MARCUS, M.D. and SIGMUND WASSERMANN, M.D., Mount Sinai Hospital. A monograph in the Bannerstone Division of American Lectures in Endocrinology, edited by Willard O. Thompson, M.D.; 156 pages; 6 illustrations; 1953. Price $4.75.

Bioassay of Anterior Pituitary and Adrenocortical Hormones. Volume V of Ciba Colloquia on Endocrinology, edited by G. E. W. WOLSTENHOLME, O.B.E., M.A., M.B., B.Ch., General Editor for the Ciba Foundation, assisted by MARGARET P. CAMERON, M.A., A.B.L.S.; 244 pages; 53 illustrations, 1953. Little, Brown and Company, Boston. Price $6.00.

Clinical Applications of Suggestion and Hypnosis, ed. 2. By WILLIAM T. HERON, M.A., Ph.D., Professor of Psychology, University of Minnesota, Minneapolis, Minnesota; 146 pages; 1953. Charles C Thomas, Publisher, Springfield, Illinois. Price $3.75.

Clinical Endocrinology, Volumes I and II. By LEWIS M. HURXTHAL, M.D., Head of the Department of Internal Medicine, Lahey Clinic, Boston; Physician, New England Baptist and New England Deaconess Hospitals, Boston, Mass.; and NATALIJA MUSULIN, B.S., M.D., Staff of Cooper Hospital, Camden, N. J.; 1620 pages; 482 figures; 146 charts; 1 color plate; 1953. J. B. Lippincott Company, Philadelphia. Price $24.00.

Comment Traiter L'Obésité. By M. ALBEAUX-FERNET, Médecin des Hôpitaux de Paris; 140 pages; 10 figures; 1953. Ernest Flammarion, Paris.

L'Année endocrinologique. Par M. ALBEAUX-FERNET, L. BELLOT, J. DANEL, J. DERIBREUX, M. GÉLINET, R. LAUMONIER, J.-D. ROMANI, J. ROBERT et J. TRUET. Cinquième année; 179 pages, 1953. Masson et Cie, Editeurs, Paris.

Le Cortex surrénal. Les bases morpho-physiologiques de sa connaissance: son role dans la pathologie, et la thérapeutique. Par A. CELESTINO DA COSTA, Professeur a la Faculté de Médecine de Lisbonne, Membre correspondant de l'Académie Nationale de Médecine de Paris et R. IRIARTE PEIXOTO, Médecin des Hôpitaux de Lisbonne; 186 pages, 26 figures; 1952. Masson et Cie, Éditeurs, Paris. 1300 Fr.

Pituitary Chromophobe Adenomas. By JOHN I. NURNBERGER, M.D., Research Associate at the Institute of Living, and Assistant Clinical Professor of Medicine (Neurology), Yale University School of Medicine, New Haven, Conn., and SAUL R. KOREY, M.D., Associate Professor of Neurology, Western Reserve School of Medicine, Cleveland, Ohio; 290 pages; 23 tables; 35 figures; 533 references; 1953. Springer Publishing Company, Inc., New York. Price $7.00.

Proceedings of the Society for the Study of Fertility. Number IV. London Conference, 1952; 65 pages; published for the Society on June 3, 1953, by W. Heffer & Sons Ltd., Cambridge, England. Price 10s.